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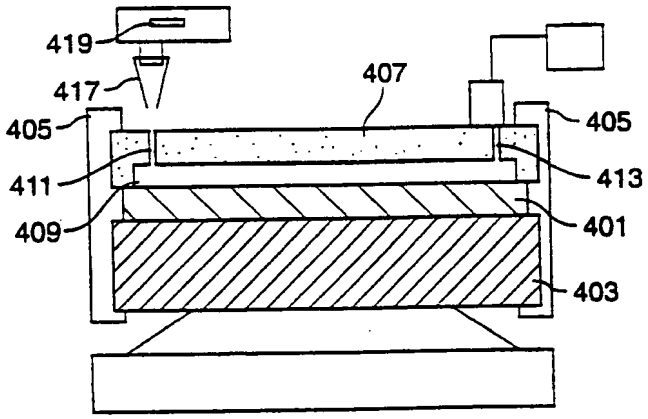


(57) Abstract

A method and device for forming large arrays of polymers on a substrate (401). According to a preferred aspect of the invention, the substrate is contacted by a channel block (407) having channels (409) therein. Selected reagents are delivered through the channels, the substrate is rotated by a rotating stage (403), and the process is repeated to form arrays of polymers on the substrate. The method may be combined with light-directed methodologies.

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# COMBINATORIAL STRATEGIES FOR POLYMER SYNTHESIS

## BACKGROUND OF THE INVENTION

This application is related to U.S. Serial No. 796,243 (filed November 22, 1991) and to U.S. Serial No. 874,849 (filed April 24, 1992), both of which are incorporated herein by reference for all purposes.

The present invention relates to the field of polymer synthesis and screening. More specifically, in one embodiment the invention provides an improved method and system for synthesizing arrays of diverse polymer sequences. According to a specific aspect of the invention, a method of synthesizing diverse polymer sequences such as peptides or oligonucleotides is provided. The diverse polymer sequences may be used, for example, in screening studies for determination of binding affinity.

Methods of synthesizing desired polymer sequences such as peptide sequences are well known to those of skill in the art. Methods of synthesizing oligonucleotides are found in, for example, Oligonucleotide Synthesis: A Practical Approach, Gate, ed., IRL Press, Oxford (1984), incorporated herein by reference in its entirety for all purposes. The so-called "Merrifield" solid phase peptide synthesis has been in common use for several years and is described in Merrifield, J. Am. Chem. Soc. (1963) 85:2149-2154, incorporated herein by reference for all purposes. Solid-phase synthesis techniques have been provided for the synthesis of several peptide sequences on, for example, a number of "pins." See e.g., Geysen et al., J. Immun. Meth. (1987) 102:259-274, incorporated herein by reference for all purposes. Other solid-phase techniques involve, for example, synthesis of various peptide sequences on different cellulose disks supported in a column. See Frank and Doring, Tetrahedron (1988) 44:6031-6040, incorporated herein by reference for all purposes. Still other solid-phase techniques are described in U.S. Patent No. 4,728,502 issued to Hamill and WO 90/00626 (Beattie, inventor).

Each of the above techniques produces only a relatively low density array of polymers. For example, the technique described in Geysen et al. is limited to producing 96 different polymers on pins spaced in the dimensions of a standard microtiter plate.

Improved methods of forming large arrays of peptides, oligonucleotides, and other polymer sequences in a short period of time have been devised. Of particular note, Pirrung et al., U.S. Patent No. 5,143,854 (see also PCT Application No. WO 90/15070) and Fodor et al., PCT Publication No. WO 92/10092, all incorporated herein by reference, disclose methods of forming vast arrays of peptides and other polymer sequences using, for example, light-

directed synthesis techniques. See also, Fodor et al., Science (1991) 251:767-777, also incorporated herein by reference for all purposes.

Some work has been done to automate synthesis of polymer arrays. For example, Southern, PCT Application No. WO 89/10977 describes the use of a conventional pen plotter to deposit three different monomers at twelve distinct locations on a substrate. These monomers were subsequently reacted to form three different polymers, each twelve monomers in length. The Southern Application also mentions the possibility of using an ink-jet printer to deposit monomers on a substrate. Further, in the above-referenced Fodor et al., PCT application, an elegant method is described for using a computer-controlled system to direct a VLSIPS™ procedure. Using this approach, one heterogenous array of polymers is converted, through simultaneous coupling at a number of reaction sites, into a different heterogenous array. This approach is referred to generally as a "combinatorial" synthesis.

The VLSIPS™ techniques have met with substantial success. However, in some cases it is desirable to have alternate/additional methods of forming polymer sequences which would not utilize, for example, light as an activator, or which would not utilize light exclusively.

## SUMMARY OF THE INVENTION

Methods and devices for synthesizing high-density arrays of diverse polymer sequences such as diverse peptides and oligonucleotides are provided by virtue of the present invention. In addition, methods and devices for delivering (and, in some cases, immobilizing) available libraries of compounds on specific regions of a substrate are provided by this invention. In preferred embodiments, various monomers or other reactants are delivered to multiple reaction sites on a single substrate where they are reacted in parallel.

According to a preferred embodiment of the invention, a series of channels, grooves, or spots are formed on or adjacent a substrate. Reagents are selectively flowed through or deposited in the channels, grooves, or spots, forming an array having different compounds - and in some embodiments, classes of compounds - at selected locations on the substrate.

According to the first specific aspect of the invention, a block having a series of channels, such as grooves, on a surface thereof is utilized. The block is placed in contact with a derivatized glass or other substrate. In a first step, a pipettor or other delivery system is used to flow selected reagents to one or more of a series of apertures connected to the channels, or place reagents in

the channels directly, filling the channels and "striping" the substrate with a first reagent, coupling a first group of monomers thereto. The first group of monomers need not be homogenous. For example, a monomer A may be placed in a first group of the channels, a monomer B in a second group of channels, and a monomer C in a third group of channels. The channels may in some embodiments thereafter be provided with additional reagents, providing coupling of additional monomers to the first group of monomers. The block is then translated or rotated, again placed on the substrate, and the process is repeated with a second reagent, coupling a second group of monomers to different regions of the substrate. The process is repeated until a diverse set of polymers of desired sequence and length is formed on the substrate. By virtue of the process, a number of polymers having diverse monomer sequences such as peptides or oligonucleotides are formed on the substrate at known locations.

According to the second aspect of the invention, a series of microchannels or microgrooves are formed on a substrate, along with an appropriate array of microvalves. The channels and valves are used to flow selected reagents over a derivatized surface. The microvalves are used to determine which of the channels are opened for any particular coupling step.

Accordingly, one embodiment of the invention provides a method of forming diverse polymer sequences on a single substrate, the substrate comprising a surface with a plurality of selected regions. The method includes the steps of forming a plurality of channels adjacent the surface, the channels at least partially having a wall thereof defined by a portion of the selected regions; and placing selected reagents in the channels to synthesize polymer sequences at the portion of the selected regions, the portion of the selected regions comprising polymers with a sequence of monomers different from polymers in at least one other of the selected regions. In alternative embodiments, the channels or flow paths themselves constitute the selected reaction regions. For example, the substrate may be a series of adjoining parallel channels, each having reaction sites therein.

According to a third aspect of the invention, a substrate is provided which has an array of discrete reaction regions separated from one another by inert regions. In one embodiment, a first monomer solution is spotted on a first set of reaction regions of a suitably derivatized substrate. Thereafter, a second monomer solution is spotted on a second set of regions, a third monomer solution is spotted on a third set and so on, until a number of the regions each have one species of monomer located therein. These monomers are reacted with the surface, and the substrate is subsequently washed and prepared for reaction with a new set of

monomers. Dimers, trimers, and larger polymers of controlled length and monomer sequence are prepared by repeating the above steps with different groupings of the reaction regions and monomer solutions. In alternative embodiments, the polymers or other compounds of the array are delivered to the regions as complete species, and thus the above polymer synthesis steps are unnecessary.

In a preferred embodiment, a plurality of reaction regions on the substrate surface are surrounded by a constraining region such as a non-wetting region which hinders the transport of reactants between adjacent reaction regions. Thus, the reactants in one region cannot flow to other regions where they could contaminate the reaction. In certain preferred embodiments, the regions of the array are defined by selective irradiation of a substrate surface containing photolabile hydrophobic protecting groups. In areas where the surface is irradiated, the hydrophobic protecting groups are removed to define reaction regions. When an aqueous or other polar reactant solution is deposited in the reaction region, it will have a relatively large wetting angle with the substrate surface so that by adjusting the amount deposited, one can ensure no flow to adjacent regions.

A further understanding of the nature and advantages of the inventions herein may be realized by reference to the remaining portions of the specification and the attached drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a generalized diagram illustrating the invention;

Fig. 2 is a flow chart illustrating the treatment steps performed in synthesizing an array of various polymers;

Fig. 3 is a mapping of a resulting array of polymers;

Fig. 4a to 4c illustrate the arrangement of three channel block templates in six process steps employed to synthesize 64 million hexapeptides from a 20 amino acid basis set;

Fig. 5a is a top view and Fig. 5b is a cross-sectional view of a first embodiment of a device used to synthesize arrays of polymer sequences;

Fig. 6 is a cross-sectional view of an embodiment containing a pressure chamber for holding a substrate against a channel block;

Figs. 7a and 7b are top views of two of two different "fanned array" channel blocks;

Fig. 8 is a cross-sectional view of a channel block and associated flow ports according to one embodiment of the invention;

Fig. 9 is a detailed cross-sectional view of the flow ports in a channel block;

Fig. 10 is a diagram of a flow system used to deliver coupling compounds and reagents to a flow cell;

Figs. 11a and 11b show an apparatus used to transfer a substrate from one channel block to another;

Fig. 12 is a diagram of a multichannel solid-phase synthesizer;

Figs. 13a and 13b illustrate alternative arrangements of the grooves in a channel block;

Fig. 14 is a schematic illustration of reaction pathways used to prepare some hydrophobic groups of the present invention;

Figs. 15a and 15b illustrate a microvalve device;

Figs. 16a and 16b illustrate an alternative embodiment of the invention;

Fig. 17 is a mapping of expected fluorescent intensities with a substrate selectively exposed to fluorescent dye.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

CONTENTS



### I. Glossary

The following terms are intended to have the following general meanings as they are used herein:

1. Ligand: A ligand is a molecule that is recognized by a receptor. Examples of ligands that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones, opiates, steroids, peptides, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, and proteins.

2. Monomer: A monomer is a member of the set of small molecules which are or can be joined together to form a polymer or a compound composed of two or more members. The set of monomers includes but is not restricted to, for example, the set of common L-amino acids, the set of D-amino acids, the set of synthetic and/or natural amino acids, the set of nucleotides and the set of pentoses and hexoses. The particular ordering of monomers within a polymer is referred to herein as the "sequence" of the polymer. As used herein, monomers refers to any member of a basis set for synthesis of a polymer. For example, dimers of the 20 naturally occurring L-amino acids form a basis set of 400 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive

steps in the synthesis of a polymer. Furthermore, each of the sets may include protected members which are modified after synthesis. The invention is described herein primarily with regard to the preparation of molecules containing sequences of monomers such as amino acids, but could readily be applied in the preparation of other polymers. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α-, β-, or ω-amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polynucleotides, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. Such polymers are "diverse" when polymers having different monomer sequences are formed at different predefined regions of a substrate. Methods of cyclization and polymer reversal of polymers are disclosed in copending application Serial No. 796,727, filed November 22, 1991, entitled "POLYMER REVERSAL ON SOLID SURFACES," incorporated herein by reference for all purposes.

3. Peptide: A peptide is a polymer in which the monomers are alpha amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. Amino acids may be the L-optical isomer or the D-optical isomer. Peptides are two or more amino acid monomers long and are often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, Biochemistry, Third Ed., 1988, which is incorporated herein by reference for all purposes.

4. Receptor: A receptor is a molecule that has an affinity for a ligand. Receptors may be naturally-occurring or manmade molecules. They can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants, viruses, cells, drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the

term receptors is used herein, no difference in meaning is intended. A "Ligand Receptor Pair" is formed when two molecules have combined through molecular recognition to form a complex.

Specific examples of receptors which can be investigated by this invention include but are not restricted to:

a) Microorganism receptors: Determination of ligands that bind to microorganism receptors such as specific transport proteins or enzymes essential to survival of microorganisms would be a useful tool for discovering new classes of antibiotics. Of particular value would be antibiotics against opportunistic fungi, protozoa, and bacteria resistant to antibiotics in current use.

b) Enzymes: For instance, a receptor can comprise a binding site of an enzyme such as an enzyme responsible for cleaving a neurotransmitter; determination of ligands for this type of receptor to modulate the action of an enzyme that cleaves a neurotransmitter is useful in developing drugs that can be used in the treatment of disorders of neurotransmission.

c) Antibodies: For instance, the invention may be useful in investigating a receptor that comprises a ligand-binding site on an antibody molecule which combines with an epitope of an antigen of interest; determining a sequence that mimics an antigenic epitope may lead to the development of vaccines in which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for autoimmune diseases (e.g., by blocking the binding of the "self" antibodies).

d) Nucleic Acids: Sequences of nucleic acids may be synthesized to establish DNA or RNA binding sequences that act as receptors for synthesized sequence.

e) Catalytic Polypeptides: Polymers, preferably antibodies, which are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products. Such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, which functionality is capable of chemically modifying the bound reactant. Catalytic polypeptides and others are described in, for example, PCT Publication No. WO 90/05746,

WO 90/05749, and WO 90/05785, which are incorporated herein by reference for all purposes.

f) Hormone receptors: Determination of the ligands which bind with high affinity to a receptor such as the receptors for insulin and growth hormone is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes or a replacement for growth hormone. Other examples of hormone receptors include the vasoconstrictive hormone receptors; determination of ligands for these receptors may lead to the development of drugs to control blood pressure.

g) Opiate receptors: Determination of ligands which bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

5. Substrate: A material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or the like. In some embodiments, the substrate itself contains wells, trenches, flow through regions, etc. which form all or part of the synthesis regions. According to other embodiments, small beads may be provided on the surface, and compounds synthesized thereon may be released upon completion of the synthesis.

6. Channel Block: A material having a plurality of grooves or recessed regions on a surface thereof. The grooves or recessed regions may take on a variety of geometric configurations, including but not limited to stripes, circles, serpentine paths, or the like. Channel blocks may be prepared in a variety of manners, including etching silicon blocks, molding or pressing polymers, etc.

7. Protecting Group: A material which is bound to a monomer unit and which may be selectively removed therefrom to expose an active site such as, in the specific example of an amino acid, an amine group. Specific examples of photolabile protecting groups are discussed in Fodor et al., PCT Publication No. WO 92/10092 (previously incorporated by reference) and U.S. Serial No. _____ filed November 2, 1992 (attorney docket No. 11509-68) incorporated herein by reference for all purposes.

8. Predefined Region: A predefined region is a localized area on a substrate which is, was, or is intended to be used for formation of a selected polymer and is otherwise referred to herein in the alternative as "reaction" region, a "selected" region, or simply a "region." The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc. In some embodiments, a predefined region and, therefore, the area upon which each distinct polymer sequence is synthesized is smaller than about 1 $cm^2$, more preferably less than 1 $mm^2$, and still more preferably less than 0.5 $mm^2$. In most preferred embodiments the regions have an area less than about 10,000 $\mu m^2$ or, more preferably, less than 100 $\mu m^2$. Within these regions, the polymer synthesized therein is preferably synthesized in a substantially pure form.

9. Substantially Pure: A polymer is considered to be "substantially pure" within a predefined region of a substrate when it exhibits characteristics that distinguish it from other predefined regions. Typically, purity will be measured in terms of biological activity or function as a result of uniform sequence. Such characteristics will typically be measured by way of binding with a selected ligand or receptor. Preferably the region is sufficiently pure such that the predominant species in the predefined region is the desired sequence. According to preferred aspects of the invention, the polymer is at least 5% pure, more preferably more than 10% to 20% pure, more preferably more than 80% to 90% pure, and most preferably more than 95% pure, where purity for this purpose refers to the ratio of the number of ligand molecules formed in a predefined region having a desired sequence to the total number of molecules formed in the predefined region.

## II. General

The invention can be used in variety of applications. For example, the invention can be used as a synthesis tool (as for example in peptide syntheses), as a screening tool (as for example in screening compound libraries for drug activity), or as a monitoring/diagnostic tool (as for example in medical or environmental testing). In one specific embodiment, the invention is used for nucleic acid-based diagnostics.

As a synthesis tool, the present invention provides for the formation of arrays of large numbers of different polymer sequences. According to a preferred embodiment, the invention provides for the synthesis of an array of different peptides or

oligonucleotides in selected regions of a substrate. Such substrates having the diverse sequences formed thereon may be used in, for example, screening studies to evaluate their interaction with receptors such as antibodies and nucleic acids. For example, in preferred embodiments the invention provides for screening of peptides to determine which if any of a diverse set of peptides has a strong binding affinity with a receptor and, in most preferred embodiments, to determine the relative binding affinity of various peptides with a receptor of interest.

Such diverse polymer sequences are preferably synthesized on a single substrate. By synthesizing the diverse polymer sequences on a single substrate, processing of the sequences to evaluate characteristics such as relative binding affinity is more easily conducted. By way of example, when an array of peptide sequences (or a library of other compounds) is to be evaluated to determine the peptides' relative binding affinity to a receptor, the entire substrate and, therefore, all or a group of the polymer sequences may be exposed to an appropriately labelled receptor and evaluated simultaneously.

In some embodiments, the present invention can be employed to localize and, in some cases, immobilize vast collections of synthetic chemical compounds or natural product extracts. In such methods, compounds are deposited on predefined regions of a substrate. The reaction of the immobilized compound (or compounds) with various test compositions such as the members of the chemical library or a biological extract are tested by dispensing small aliquots of each member of the library or extract to a different region. Competitive assays or other well-known techniques can be used to identify a desired activity. As an example, a large collection of human receptors is deposited on a substrate, one in each region to form an array. A plant/animal extract is then screened for binding to various receptors of the array.

The present invention has certain features in common with the "light directed" methods described in U.S. Patent No. 5,143,854, previously incorporated by reference. The light directed methods discussed in the '854 patent involve activating predefined regions of the substrate and then contacting the substrate with a preselected monomer solution. The predefined regions can be activated with a light source shown through a mask (much in the manner of photolithography techniques used in integrated circuit fabrication). Other regions of the substrate remain inactive because they are blocked by the mask from illumination. Thus, a light pattern defines which regions of the substrate react with a given monomer. By repeatedly activating different sets of predefined regions and contacting different monomer solutions with the substrate, a diverse

array of polymers is produced on the substrate. Of course, other steps such as washing unreacted monomer solution from the substrate can be used as necessary.

In the present invention, a mechanical device or physical structure defines the regions which are available to react with a given monomer. In some embodiments, a wall or other physical barrier is used to block a given monomer solution from contacting any but a few selected regions of a substrate. In other embodiments, the amount of the monomer (or other) solution deposited and the composition of the substrate act to separate different monomer solutions on the substrate. This permits different monomers to be delivered and coupled to different regions simultaneously (or nearly simultaneously) and reduces the number of separate washing and other reaction steps necessary to form an array of polymers. Further, the reaction conditions at different activated regions can be controlled independently. Thus, the reactant concentrations and other parameters can be varied independently from reaction site to reaction site, to optimize the procedure.

In alternative preferred embodiments of the present invention, light or another activator is used in conjunction with the physical structures to define reaction regions. For example, a light source activates various regions of the substrate at one time and then a mechanical system directs monomer solutions to different activated regions, in parallel.

III. Methods for Mechanical Delivery of Reagents

In preferred embodiments of the present invention, reagents are delivered to the substrate by either (1) flowing within a channel defined on predefined regions or (2) "spotting" on predefined regions. However, other approaches, as well as combinations of spotting and flowing, may be employed. In each instance, certain activated regions of the substrate are mechanically separated from other regions when the monomer solutions are delivered to the various reaction sites.

A typical "flow channel" method of the present invention can generally be described as follows. Diverse polymer sequences are synthesized at selected regions of a substrate by forming flow channels on a surface of the substrate through which appropriate reagents flow or in which appropriate reagents are placed. For example, assume a monomer "A" is to be bound to the substrate in a first group of selected regions. If necessary, all or part of the surface of the substrate in all or a part of the selected regions is activated for binding by, for example, flowing appropriate reagents through all or some of the channels, or by washing the entire substrate with appropriate reagents. After placement of a channel

block on the surface of the substrate, a reagent having the monomer A flows through or is placed in all or some of the channel(s). The channels provide fluid contact to the first selected regions, thereby binding the monomer A on the substrate directly or indirectly (via a linker) in the first selected regions.

Thereafter, a monomer B is coupled to second selected regions, some of which may be included among the first selected regions. The second selected regions will be in fluid contact with a second flow channel(s) through translation, rotation, or replacement of the channel block on the surface of the substrate; through opening or closing a selected valve; or through deposition of a layer of photoresist. If necessary, a step is performed for activating at least the second regions. Thereafter, the monomer B is flowed through or placed in the second flow channel(s), binding monomer B at the second selected locations. In this particular example, the resulting sequences bound to the substrate at this stage of processing will be, for example, A, B, and AB. The process is repeated to form a vast array of sequences of desired length at known locations on the substrate.

After the substrate is activated, monomer A can be flowed through some of the channels, monomer B can be flowed through other channels, a monomer C can be flowed through still other channels, etc. In this manner, many or all of the reaction regions are reacted with a monomer before the channel block must be moved or the substrate must be washed and/or reactivated. By making use of many or all of the available reaction regions simultaneously, the number of washing and activation steps can be minimized.

Various embodiments of the invention will provide for alternative methods of forming channels or otherwise protecting a portion of the surface of the substrate. For example, according to some embodiments, a protective coating such as a hydrophilic or hydrophobic coating (depending upon the nature of the solvent) is utilized over portions of the substrate to be protected, sometimes in combination with materials that facilitate wetting by the reactant solution in other regions. In this manner, the flowing solutions are further prevented from passing outside of their designated flow paths.

The "spotting" embodiments of the present invention can be implemented in much the same manner as the flow channel embodiments. For example, a monomer A can be delivered to and coupled with a first group of reaction regions which have been appropriately activated. Thereafter, a monomer B can be delivered to and reacted with a second group of activated reaction regions. Unlike the flow channel embodiments described above, reactants are delivered by directly depositing (rather than flowing) relatively

small quantities of them in selected regions. In some steps, of course, the entire substrate surface can be sprayed or otherwise coated with a solution. In preferred embodiments, a dispenser moves from region to region, depositing only as much monomer as necessary at each stop. Typical dispensers include a micropipette to deliver the monomer solution to the substrate and a robotic system to control the position of the micropipette with respect to the substrate. In other embodiments, the dispenser includes a series of tubes, a manifold, an array of pipettes, or the like so that various reagents can be delivered to the reaction regions simultaneously.

IV. Flow Channel Embodiments

Fig. 1 illustrates an example of the invention. In this particular example, monomers and dimers of the monomer group A, B, C, and D are to be bound at selected regions of the substrate. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place.

The substrate and its surface form a support on which to carry out the reactions described herein. These monomers are bound using first flow channel paths $x_1$, $x_2$, $x_3$, and $x_4$ which are formed or placed on or adjacent the substrate in a first orientation, and second flow channel paths $y_1$, $y_2$, $y_3$, and $y_4$ which are formed or placed on or adjacent the substrate in a second orientation. The second flow channel paths intersect at least a part of the first flow channel paths. The flow channels are formed according to techniques which are described in greater detail elsewhere herein.

Initially the substrate is subjected to one or more preliminary treatments such as, for example, cleaning and the optional placement of "linker" molecules on the surface thereof. The substrate may also be provided with various active groups, common monomer sequences which will form a part of the polymers, or the like.

Thereafter, in a first coupling step, one or more of the flow channels are provided with the first monomer A, which binds through covalent bonds or otherwise to the substrate (directly or indirectly) where the flow channel contacts the substrate. In the particular example shown in Fig. 1, the flow channels $x_1$ and $x_2$ are utilized, binding the monomer A to the substrate along the entire

length of the substrate adjacent to the $x_1$ and $x_2$ channels. Each coupling step may in some embodiments be composed of a variety of substeps. For example, each coupling step may include one or more substeps for washing, chemical activation, or the like.

Thereafter or concurrently therewith, as shown in Fig. 2, a second monomer B is provided to selected flow channels and the monomer B binds to the substrate where the second flow channels provide contact therewith. In the particular example shown in Fig. 2, monomer B is bound along channels $x_3$ and $x_4$. When the monomers A and B flow through their respective flow channels simultaneously, only a single process step is required to perform two coupling steps simultaneously. As used herein, a "process step" refers to the injection of one or more channels with one or more reagents. A "coupling step" refers to the addition of a monomer in a polymer.

Processing thereafter continues in a similar manner with monomers C and D in the manner shown in the flow diagram of Fig. 2, with monomer C being bound in the flow channels $y_1$ and $y_2$, and D being bound in the flow channels $y_3$ and $y_4$. Preferably, monomers C and D are directed through the flow channels $y_1$ to $y_4$ simultaneously whereby two coupling steps are performed with a single process step. Light regions in Fig. 1 indicate the intersections of the resulting flow paths.

Fig. 3 illustrates the mapping of sequences formed using the above illustrated steps. As shown therein, the sequences A, B, C, D, AD, BD, AC, and BC have been formed using only two process steps. Accordingly, it is seen that the process provides for the synthesis of vast arrays of polymer sequences using only a relatively few process steps. By way of further example, it is necessary to use only two process steps to form all of the $4^2 = 16$ dimers of a four-monomer basis set. By way of further example, to form all $4^8$ octomers of a four-monomer basis set, it is necessary to provide only 256 flow channels oriented in the "x" direction, and 256 flow channels oriented in the "y" direction, with a total of eight coupling steps.

The power of the technique is further illustrated by synthesizing the complete array of six hexamer peptides from a 20 amino acid basis set. This array will include $20^6$ or 64,000,000 regions defining 64,000,000 different peptides and can be formed in only six process steps. Further, the method requires only three different templates, one having 20 parallel channels, a second having 400 channels each 1/20th as wide as the first, and a third having 8000 channels each 1/20th as wide as the second. Each template will be used in two process steps, each at an orientation at 90 degrees with respect to the other as illustrated in Fig. 4. With the first

template, the substrate is activated and then solutions of each of the 20 amino acid basis set (or other 20 member basis set) are flowed over and reacted on a different predefined stripe in a first orientation. This is the first process step and includes 20 coupling or attachment steps, which can be performed simultaneously. Next, the entire substrate is again activated and the first template is placed in a second orientation, perpendicular to the first (Fig. 4a). The 20 amino acid solutions are then flowed along 20 new predefined stripes (each perpendicular to the original set of stripes). In each of these two process steps, the 20 predefined regions (the stripes along the flow channels) are first activated and then contacted with the individual monomers so that all 20 stripes are reacted before the next activation step is necessary. In other words, 20 coupling steps are conducted in parallel, greatly reducing the number of necessary activation steps.

The four remaining coupling steps employ the second and third templates. In the third and fourth process steps (Fig. 4b), 20 channels are devoted to each monomer, and in the fifth and sixth process steps (Fig. 4c), 400 channels are devoted to each monomer. As with the first two steps, the entire substrate undergoes reaction during a single process step. Thus, only six process steps (requiring a total of about 24 hours) are required to produce the entire library of 64,000,000 peptide hexamers. In a different embodiment, a single template having 8000 channels to control delivery (e.g. 400 channels for each of the 20 amino acids in the first round) can produce the full library of hexamers with only a single rotation step. Thus, the present invention offers extremely rapid methods of preparing diverse polymer arrays.

Figs. 5a and 5b illustrate details of a first embodiment of a device used for performing the synthesis steps described above. In particular, Fig. 5a illustrates the device in top view, while Fig. 5b illustrates the device in cross-sectional side view. In the particular embodiment shown in Fig. 5, the device is used to synthesize polymer sequences on substrate 401. Substrate 401 is coupled to a rotating stage 403 and removably held by clamp 405 to channel block 407. Channel block 407 has etched therein a plurality of channels 409 in the form of stripes therein. Each channel is provided with a flow inlet 411 and an outlet 413. A vacuum source 415 is applied to one or more of the outlets 413, while a pipettor 417 is slidably mounted on arm 419 to deliver selected reagents from reservoir(s) 421 to selected flow inlets 411.

The details of a second preferred embodiment are shown in Figs. 6-11. Fig. 6 displays an apparatus for holding a substrate 111 in place against a channel block 109 by evenly distributing pressure over the substrate in a pressure chamber 101. Pressurized gas is

admitted through gas pressure inlet 103 to provide clamping pressure to immobilize the substrate while fluids are flowed from fluid flow inlet 115, through channel 123, and out fluid outlet 117. The upper and lower portions of the pressure chamber housing 105 and 125 are held together by nuts 121 and bolts 104. Of course, other means such as clamps can be used to hold the pressure chamber housing portions together.

Fig. 7 illustrates preferred flow path configurations in channel blocks of the present invention. As shown in Figs. 7a, fluid delivery sites 127, 129, 131, 133, 135, and 137 are connected to channels leading to reaction region 141. A similar arrangement is shown for comparison in Fig. 7b where the orientation of the flow channels in the reaction regions is shifted by 90 degrees on a rectangular channel block. Vacuum ports 145 and 146 to an external vacuum line are provided so that substrate position is maintained during fluid flow.

The channels shown in Figs. 7a and 7b form a "fanned channel array" on channel block 139 in a manner analogous to that of the lead pattern employed in integrated circuits. This provides significantly increased separation of fluid delivery points in comparison to the high density of channels in the reaction region. In a 2 inch by 3 inch substrate, at least about a 4:1 increase in spatial separation typically can be attained by the fanned arrangement. Thus, if the channels in the reaction regions are separated by 200 microns, the delivery ports can be separated by 0.8 mm.

The spatial separation can be further increased by staggering the delivery ports as shown for ports 127, 129, and 131. This can provide an additional channel separation of at least about 3:1. Thus, for the channels separated by 200 microns, a staggered fanned array provides 2.4 mm separation between the delivery ports. Thus, fluid can be delivered to a high-density array of channels in the reaction region from standard 1.6 mm Teflon™ tubing. If additional spacing is necessary, the substrate size can be increased, while preserving the reaction region size.

As shown in Fig. 8, the fluid delivery ports are accessed from holes in the back surface of a stabilizing plate 108 on the channel block. The stabilizing plate, which is preferably made from fused pyrex, provides structural integrity to the channel block during clamping in the pressure chamber. It may also provide a means to access the channel block ports and reduce leakage between ports or channels. In preferred embodiments, the channels 123 of the channel block are formed on a wafer 106 which generally may be any machinable or cast material, and preferably may be etched silicon or a micromachined ceramic. In other embodiments, the channel block is

pressure-formed or injection-molded from a suitable polymer material. The entire channel block arrangement is mounted on a rigid channel block sub-plate 110 including a vacuum line 112, ports for fluid delivery lines 115, ports for fluid outlet lines 117, and recessed regions for plug ends 151 and 153. With this arrangement, the substrate can be clamped against the top surface of the channel block (by vacuum or pressurized gas as shown in the embodiment of Fig. 6) while fluid enters and exits from below. Preferably, the subplate will be made from a rigid material such as stainless steel or anodized aluminum.

Individual micro tubing connections can be made for each channel as shown in Fig. 9. Plug ends 151 are provided with a conical upper surface that mates with a conical recess 118 in pyrex stabilizing plate 108. Plug ends 151 also have a cylindrical lower surface that mates with cylindrical recess 116 in sub-plate 110. The subplate and stabilizing plate are held together by bolt 114 and threaded insert 112 or other suitable engagement means.

Fig. 10 shows a fluid flow diagram of a preferred system of the present invention. The pressure is controlled at point 25 (P1) and point 21 (P2) so that a pressure drop (P1-P2) is maintained across the system. Coupling compounds such as activated monomers are supplied from reservoirs 31, 32, and 33. Additional reagents are supplied from reservoirs 15, 17, and 19. Of course, the monomer and coupling reagent reservoirs shown in Fig. 10 are representative of a potentially much larger series of reservoirs. The reagents and coupling compounds are combined at nodes 27, 28, and 29 before being directed to channel block 139. Mixing of the appropriate reagents and coupling compounds is controlled by valves at the nodes which are in turn controlled by electronic control 23. Waste fluids that have been directed across the substrate are removed through line 35.

The system displayed in Fig. 10 allows control of all channels in parallel by regulating only a few variables. For example, a constant pressure gradient is maintained across all channels simultaneously by fixing P1 and P2. Thus, the flow rate in each channel is dependent upon the cross-sectional area of the flow channel and the rheological properties of the fluids. Because the channels have a uniform cross-section and because the coupling compounds are typically provided as dilute solutions of a single solvent, a uniform flow rate is created across all channels. With this system the coupling time in all channels can be varied simultaneously by simply adjusting the pressure gradient across the system. The valves of the system are preferably controlled by a single electronic output from control 23.

The fanned channel array design shown in Fig. 7 provides for two separate channel blocks to be used in successive process

steps during a chemical synthesis. One block forms a horizontal array on the solid substrate, while the other block forms a vertical array. To create a matrix of intersecting rows and columns of chemical compounds, the solid substrate is transferred from one block to the other during successive process steps. While many experiments require only a single transfer from one block to the other during a series of process steps, the fanned channel array transfer block 75 illustrated in Figs. 11a and 11b provides one device for maintaining accurate registration of the solid substrate 71 relative to the channel blocks 79 during repeated transfers. In some embodiments, a single channel block can be used for horizontal and vertical arrays by simply rotating it by 90 degrees as necessary.

The transfer block is positioned with respect to the channel block so that the dimensional characteristics of the solid substrate are not used in the alignment. The transfer block 75 is aligned to the channel block by kinematic mount 81 while vacuum is switched from vacuum line 83 on the channel block to vacuum line 77 on the transfer block (during normal operation, a vacuum holds the substrate against the channel block). The substrate and transfer block are then moved and repositioned relative to the second channel bock. Vacuum is then switched to the second channel block, retaining the substrate in proper alignment. This way, accurate registration can be assured between process steps regardless of variation in the dimensions of individual substrates. The transfer block system also maintains alignment of the matrix area during transfers to and from the flow cell during experiments utilizing both mechanical and light-directed process steps.

In some embodiments the channel block need not be utilized. Instead, in some embodiments, small "strips" of reagent are applied to the substrate by, for example, striping the substrate or channels therein with a pipettor. Such embodiments bear some resemblance to the spotting embodiments of this invention. According to other embodiments the channels will be formed by depositing a photoresist such as those used extensively in the semiconductor industry. Such materials include polymethyl methacrylate (PMMA) and its derivatives, and electron beam resists such as poly(olefin sulfones) and the like (more fully described in Ghandi, "VLSI Fabrication Principles," Wiley (1983) Chapter 10, incorporated herein by reference in its entirety for all purposes). According to these embodiments, a resist is deposited, selectively exposed, and etched, leaving a portion of the substrate exposed for coupling. These steps of depositing resist, selectively removing resist and monomer coupling are repeated to form polymers of desired sequence at desired locations.

In some embodiments, a resist can be used to activate certain regions of the substrate. Certain resist materials such as acid-generating polymers, for example, will release protons upon irradiation. According to these embodiments, a substrate covered with such material is irradiated through a mask or otherwise selectively irradiated so that the irradiated regions of the substrate are exposed to acidic conditions. Acid-labile protecting group on the substrate or oligomers on the substrate are removed, leaving an activated region. At this point, all or part of the resist may be removed. In preferred embodiments, the resist will be removed only in the activated regions, so that the channels are formed at the activated regions. Alternatively, the resist can be removed from the entire substrate. In this case, a separate channel block can then be contacted with the substrate to define flow channels, or a conventional VLSIPS™ procedure can be employed.

In preferred embodiments, the substrate is conventional glass, pyrex, quartz, any one of a variety of polymeric materials, or the like. Of course, the substrate may be made from any one of a variety of materials such as silicon, polystyrene, polycarbonate, or the like. In preferred embodiments the channel block is made of silicon or polychlorotrifluorethylene, such as material known under the trade name KelF™ 80 made by 3M, although a wide variety of materials such as polystyrene, polycarbonate, glass, elastomers such as Kalrez made by DuPont, various ceramics, stainless steel, or the like may be utilized.

The channels in the channel block are preferably made by machining, compression molding, injection molding, lithography, laser cutting, or the like depending upon the material of interest. In some embodiments employing larger channel blocks, the raised portions of the channels in the channel block are treated by lapping with lapping film (0.3 μm grit). Such smooth surfaces provide good seals to the substrate without the use of a sealant and, therefore, without the possibility of leaving sealant material on the substrate when rotating the channel block. Preferably, all operations are conducted at substantially ambient temperatures and pressures.

A particularly preferred channel block is prepared by chemical etching of polished silicon wafers. Chemical etching is a widely used technique in integrated circuit fabrications. It can easily provide 60 or more 100 micron channels on a 12.8 mm region of a polished silicon wafer. Even after etching, the top (unetched) surface regions of the wafer retains the very flat profile of the unetched wafer. Thus, close contact with the substrate is ensured during flow cell operation.

In operation, the surface of the substrate is appropriately treated by cleaning with, for example, organic

solvents, methylene chloride, DMF, ethyl alcohol, or the like. Optionally, the substrate may be provided with appropriate linker molecules on the surface thereof. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing from 2-10 monomers or more, diamines, diacids, amino acids, or combinations thereof. Thereafter, the surface is provided with protected surface active groups such as TBOC or FMOC protected amino acids. Such techniques are well known to those of skill in the art.

Thereafter, the channel block and the substrate are brought into contact forming fluid-tight channels bounded by the grooves in the channel block and the substrate. When the channel block and the substrate are in contact, a protecting group removal agent is, thereafter, directed through a first selected channel or group of channels by placing the pipettor on the flow inlet of the selected channel and, optionally, the vacuum source on the outlet of the channel. In the case of, for example, TBOC protected amino acids, this protecting group removal agent may be, for example, trifluoroacetic acid (TFA). This step is optionally followed by steps of washing to remove excess TFA with, for example, dichloromethane (DCM).

Thereafter, a first amino acid or other monomer A is directed through the first selected flow channel. Preferably this first amino acid is also provided with an appropriate protecting group such as TBOC, FMOC, NVOC, or the like. This step is also followed by appropriate washing steps. The of deprotection/coupling steps employed in the first group of channels are concurrently with or thereafter repeated in additional groups of channels. In preferred embodiments, monomer A will be directed through the first group of channels, monomer B will be directed through a second group of flow channels, etc., so that a variety of different monomers are coupled on parallel channels of the substrate.

Thereafter, the substrate and the channel block are separated and, optionally, the entire substrate is washed with an appropriate material to remove any unwanted materials from the points where the channels contact the substrate.

The substrate and/or block is then, optionally, washed and translated and/or rotated with the stage. In preferred embodiments, the substrate is rotated 90 degrees from its original position, although some embodiments may provide for greater or less rotation, such as from 0 to 180 degrees. In other embodiments, such as those discussed in connection with the device shown in Fig. 7, two or more different channel blocks are employed to produce different flow patterns across the substrate. When the channel block is rotated, it may simultaneously be translated with respect to the substrate. "Translated" means any relative motion of the substrate

and/or channel block, while "rotation" is intended to refer to rotation of the substrate and/or channel block about an axis perpendicular to the substrate and/or channel block. According to some embodiments the relative rotation is at different angles for different stages of the synthesis.

The steps of deprotection, and coupling of amino acids or other monomers is then repeated, resulting in the formation of an array of polymers on the surface of the substrate. For example, a monomer B may be directed through selected flow channels, providing the polymer AB at intersections of the channels formed by the channel block in the first position with the channels formed by the channel block after 90-degree rotation.

While rotation of the channel block is provided according to preferred embodiments of the invention, such rotation is not required. For example, by simply flowing different reagents through the channels, polymers having different monomer sequences may be formed. Merely by way of a specific example, a portion of the channels may be filled with monomer "A," and a portion filled with monomer "B" in a first coupling step. All or a portion of the first channels are then filled with a monomer "C," and all or a portion of the second channels are filled with a monomer "D," forming the sequences AB and CD. Such steps could be used to form 100 sequences using a basis set of 10 monomers with a 100-groove channel block.

In another embodiment, the invention provides a multichannel solid-phase synthesizer as shown in Fig. 12. In this embodiment, a collection of delivery lines such as a manifold or collection of tubes 1000 delivers activated reagents to a synthesis support matrix 1002. The collection of tubes 1000 may take the form of a rigid synthesis block manifold which can be precisely aligned with the synthesis support matrix 1002. The support matrix contains a plurality of reaction regions 1004 in which compounds may be immobilized or synthesized. In preferred embodiments, the reaction regions include synthesis frits, pads, resins, or the like.

The solutions delivered to the individual reactant regions of the support matrix flow through the reaction regions to waste disposal regions, recycling tank(s), separators, etc. In some embodiments, the reaction solutions simply pass through the reaction regions under the influence of gravity, while in other embodiments, the solutions are pulled or pushed through the reaction regions by vacuum or pressure.

The individual reaction regions 1004 of the support matrix are separated from one another by walls or gaskets 1006. These prevent the reactant solution in one reaction region from moving to and contaminating adjacent reaction regions. In one embodiment, the reaction regions are defined by tubes which may be

filled with resin or reaction mixture. The gasketing allows close contact between the support matrix 1002 and a "mask" (not shown). The mask serves to control delivery of a first group reactant solutions through predetermined lines (tubes) to a first set of reaction regions. By ensuring close contact between the delivery tubes 1000, the mask, and the support matrix 1002, the probability that reaction solutions will be accidently added to the wrong reaction site is reduced.

After each process step, the mask can be changed so that a new group reactants is delivered to a new set of reaction regions. In this manner, a combinatorial strategy can be employed to prepare a large array of polymers or other compounds. In other embodiments, mechanisms other than masks can be employed to block the individual delivery tubes. For example, an array of control valves within the tubes may be suitable for some embodiments.

By adjusting the thickness of the synthesis support matrix, the quantity of immobilized material in the reaction regions can be controlled. For example, relatively thin support synthesis matrices can be used to produce small amounts of surface bound oligomers for analysis, while thicker support matrices can be used to synthesize relatively large quantities of oligomers which can be cleaved from the support for further use. In the latter embodiment, a collector having dimensions matching the individual synthesis supports can be employed to collect oligomers that are ultimately freed from the reaction matrix.

To illustrate the ability of this system to synthesize numerous polymers, a square synthesis matrix measuring 10 cm along each side and having 5 mm reaction regions separated by 5 mm wide gaskets provides 100 individual syntheses sites (reaction regions). By reducing the size of the reaction regions to 2.5 mm on each side, 400 reactions regions become available.

While linear grooves are shown herein in the preferred aspects of the invention, other embodiments of the invention will provide for circular rings or other shapes such as circular rings with radial grooves running between selected rings. According to some embodiments, channel blocks with different geometric configurations will be used from one step to the next, such as circular rings in one step and linear stripes in the next. Fig. 13a illustrates one of the possible arrangements in which the channels 409 are arranged in a serpentine arrangement in the channel block 407. Through appropriate translation and/or rotation of the channel block, polymers of desired monomer sequence are formed at the intersection of the channels during successive polymer additions, such as at location 501, where the intersection of a previous or subsequent set of channels is shown in dashed lines. Fig. 13b

illustrates another arrangement in which channels (in this case without flow paths 413) are provided in a linear arrangement, with groups 503 and 505 located in adjacent regions of the substrate and extending only a portion of the substrate length.

In some embodiments of the invention, the various reagents, such as those containing the various monomers, are not pumped through the apertures 413. Instead, the reagent is placed in one of the grooves, such as the grooves 409 shown in Fig. 13b, filling the groove. The substrate is then placed on top of the channel block, and the exposed portions of the substrate are permitted to react with the materials in the grooves. In preferred embodiments, the channels are of the same width as the raised regions between the channels. According to these embodiments, the substrate may then be moved laterally by one channel width or an integer multiple of a channel width, permitting reaction with and placement of monomers on the regions between the channels in a previous coupling step. Thereafter, the substrate or channel block will be rotated for the next series of coupling steps.

In preferred embodiments, the process is repeated to provide more than 10 different polymer sequences on the surface of the substrate. In more preferred embodiments, the process is repeated to provide more than $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, or more polymer sequences on a single substrate. In some embodiments the process is repeated to provide polymers with as few as two monomers, although the process may be readily adapted to form polymers having 3, 4, 5, 6, 10, 15, 20, 30, 40, 50, 75, 100 or more monomers therein.

According to preferred embodiments, the array of polymer sequences is utilized in one or more of a variety of screening processes, one of which is described in copending application U.S. Serial No. 796,947, filed on November 22, 1991 and incorporated herein by reference for all purposes. For example, according to one embodiment, the substrate is then exposed to a receptor of interest such as an enzyme or antibody. According to preferred embodiments, the receptor is labelled with fluorescein, or otherwise labelled, so as to provide for easy detection of the location at which the receptor binds. According to some embodiments, the channel block is used to direct solutions containing a receptor over a synthesized array of polymers. For example, according to some embodiments the channel block is used to direct receptor solutions having different receptor concentrations over regions of the substrate.

According to most preferred embodiments, amplification of the signal provided by way of fluorescein labelling is provided by exposing the substrate to the antibody of interest, and then exposing the substrate to a labelled material which is complementary to the antibody of interest and which preferably binds at multiple

locations of the antibody of interest. For example, in one specific embodiment, if a mouse antibody is to be studied, a labelled second antibody may be exposed to the substrate which is, for example, goat antimouse. Such techniques are described in PCT Publication No. WO92/10092, previously incorporated herein by reference.

V. Spotting Embodiments

According to some embodiments, monomers (or other reactants) are deposited from a dispenser in droplets that fill predefined regions. For example, in a single coupling step, the dispenser deposits a first monomer in a series of predefined regions by moving over a first region, dispensing a droplet, moving to a second region, dispensing a droplet, and so on until the each of the selected regions has received the monomer. Next the dispenser deposits a second monomer in a second series of predefined regions in much the same manner. In some embodiments, more than one dispenser may be used so that more than one monomer are simultaneously deposited. The monomers may react immediately on contact with the reaction regions or may require a further activation step, such as the addition of catalyst. After some number of monomers have been deposited and reacted in predefined regions throughout the substrate, the unreacted monomer solution is removed from the substrate. This completes a first process step.

For purposes of this embodiment, the spacing between the individual reaction regions of the substrate preferably will be less than about 3 mm, and more preferably between about 5 and 100 $\mu$m. Further, the angular relation between the regions is preferably consistent to within 1 degree and more preferably to within 0.1 degree. Preferably, the substrate will include at least about 100 reaction regions, more preferably at least about 1000 reaction regions, and most preferably at least about 10,000 reaction regions. Of course, the density of reaction regions on the substrate will vary. In preferred embodiments, there are at least about 1000 reaction regions per $cm^2$ of substrate, and more preferably at least about 10,000 regions per $cm^2$.

To deposit reactant droplets consistently at precisely specified regions, a frame of reference common to the delivery instrument and the substrate is required. In other words, the reference coordinates of the instrument must be accurately mapped onto the reference coordinates of the substrate. Ideally, only two reference points on the substrate are necessary to map the array of polymer regions completely. The dispenser instrument locates these reference points and then adjusts its internal reference coordinates to provide the necessary mapping. After this, the dispenser can move a particular distance in a particular direction and be positioned

directly over a known region. Of course, the dispenser instrument must provide precisely repeatable movements. Further, the individual regions of the array must not move with respect to the reference marks on the substrate after the reference marks have been formed. Unfortunately, pressing or other mechanical operations commonly encountered during fabrication and use of a substrate can warp the substrate such that the correspondence between the reference marks and the reaction regions is altered.

Thus, in preferred embodiments, a substrate containing both "global" and "local" reference marks is employed. In preferred embodiments, two global reference marks are conveniently located on the substrate to define the initial frame of reference. When these points are located, the dispenser instrument has an approximate map of the substrate and the predefined regions therein. To assist in locating the exact position of the regions, the substrate is further subdivided into local frames of reference. Thus, in an initial, "course" adjustment, the dispenser is positioned within one of the local frames of reference. Once in the local region, the dispensing instrument looks for local reference marks to define further a local frame of reference. From these, the dispenser moves exactly to the reaction region where the monomer is deposited. In this manner, the effects of warpage or other deformation can be minimized. The number of local reference marks is determined by the amount of deformation expected in the substrate. If the substrate is sufficiently rigid so that little or no deformation will occur, very few local reference marks are required. If substantial deformation is expected, however, more local reference marks are required.

In order to locate the appropriate reference point initially and align the dispenser with respect to it, a vision or blind system may be employed. In a vision system, a camera is rigidly mounted to the dispenser nozzle. When the camera locates the reference point(s), the dispenser is known to be a fixed distance and direction away from the point, and a frame of reference is established. Blind systems of the present invention locate the reference point(s) by capacitive, resistive, or optical techniques, for example. In one example of an optical technique, a laser beam is transmitted through or reflected from the substrate. When the beam encounters a reference mark, a change in light intensity is detected by a sensor. Capacitive and resistive techniques are similarly applied. A sensor registers a change in capacitance or resistivity when a reference point is encountered.

Starting at a single reference point, the dispenser is translated from one reaction region to other regions of the substrate by a correct distance in the correct direction (this is the "dead reckoning" navigational technique). At each stop, the dispenser

deposits correctly metered amounts of monomer. Analogous systems widely used in the microelectronic device fabrication and testing arts can move at rates of up to 3-10 stops per second. The translational (X-Y) accuracy of such systems is well within 1 μm.

Translational mechanisms for moving the dispenser are preferably equipped with closed loop position feedback mechanisms (encoders) and have insignificant backlash and hysteresis. In preferred embodiments, the translation mechanism has a high resolution, i.e. better than one motor tick per encoder count. Further, the electro-mechanical mechanism preferably has a high repeatability relative to the reaction region diameter travel distance (typically ± 1μm or better).

To deposit a drop of monomer solution on the substrate accurately, the dispenser nozzle must be placed a correct distance above the surface. In one embodiment, the dispenser tip preferably will be located about 5-50 μm above the substrate surface when a five nanoliter drop is released. More preferably, the drop will be about 10 μm above the substrate surface when the drop is released. The degree of control necessary to achieve such accuracy is attained with a repeatable high-resolution translation mechanism of the type described above. In one embodiment, the height above the substrate is determined by moving the dispenser toward the substrate in small increments, until the dispenser tip touches the substrate. At this point, the dispenser is moved away from the surface a fixed number of increments which corresponds to a specific distance. From there the drop is released to the cell below. Preferably, the increments in which the dispenser moves less than about 5 μm and more preferably less than about 2 μm.

In an alternative embodiment, the dispenser nozzle is encircled by a sheath that rigidly extends a fixed distance beyond the dispenser tip. Preferably, this distance corresponds to the distance the solution drop will fall when delivered to the selected reaction region. Thus, when the sheath contacts the substrate surface, the movement of the dispenser is halted and the drop is released. It is not necessary in this embodiment to move the dispenser back, away from the substrate, after contact is made. The point of contact with the surface can be determined by a variety of techniques such as by monitoring the capacitance or resistance between the tip of the dispenser (or sheath) and the substrate below. A rapid change in either of these properties is observed upon contact with the surface.

To this point, the spotting system has been described

only in terms of translational movements. However, other systems may also be employed. In one embodiment, the dispenser is aligned with respect to the region of interest by a system analogous to that employed in magnetic and optical storage media fields. For example, the region in which monomer is to be deposited is identified by a track and sector location on the disk. The dispenser is then moved to the appropriate track while the disk substrate rotates. When the appropriate cell is positioned below the dispenser (as referenced by the appropriate sector on the track), a droplet of monomer solution is released.

Control of the droplet size may be accomplished by various techniques. For example, in one embodiment, a conventional micropipetting instrument is adapted to dispense droplets of five nanoliters or smaller from a capillary. Such droplets fit within regions having diameters of 300 μm or less when a non-wetting mask of the invention is employed.

In another embodiment, the dispenser is a piezoelectric pump that generates charged droplets and guides them to the reaction region by an electric field in a manner analogous to conventional ink-jet printers. In fact, some ink-jet printers can be used with minor modification by simply substituting a monomer containing solution for ink. For example, Wong et al., European Patent Application 260 965, incorporated herein by reference for all purposes, describes the use of a commercial printer to apply an antibody to a solid matrix. In the process, a solution containing the antibody is forced through a small bore nozzle that is vibrating in a manner that fragments the solution into discrete droplets. The droplets are subsequently charged by passing through an electric field and then deflected onto the matrix material.

A conventional ink drop printer includes a reservoir in which ink is held under pressure. The ink reservoir feeds a pipe which is connected to a nozzle. An electromechanical transducer is employed to vibrate the nozzle at some suitable high frequency. The actual structure of the nozzle may have a number of different constructions, including a drawn glass tube which is vibrated by an external transducer, or a metal tube vibrated by an external transducer (e.g. a piezoelectric crystal) or a magnetostrictive metal tube which is magnetostrictively vibrated. The ink accordingly is ejected from the nozzle in a stream which shortly thereafter breaks into individual drops. An electrode may be present near the nozzle to impart a charge to the droplets. Conventional ink drop dispensers are described in U.S. Patent Nos. 3,281,860 and 4,121,222, which are incorporated by reference herein for all purposes.

In a different preferred embodiment, the reactant solutions are delivered from a reservoir to the substrate by an

electrophoretic pump. In this device, a thin capillary connects a reservoir of the reactant with the nozzle of the dispenser. At both ends of the capillary, electrodes are present to provide a potential difference. As is known in the art, the speed at which a chemical species travels in a potential gradient of an electrophoretic medium is governed by a variety of physical properties, including the charge density, size, and shape of the species being transported, as well as the physical and chemical properties of the transport medium itself. Under the proper conditions of potential gradient, capillary dimensions, and transport medium rheology, a hydrodynamic flow will be set up within the capillary. Thus, in an electrophoretic pump of the present invention, bulk fluid containing the reactant of interest is pumped from a reservoir to the substrate. By adjusting the appropriate position of the substrate with respect to the electrophoretic pump nozzle, the reactant solution is precisely delivered to predefined reaction regions.

In one particularly useful application, the electrophoretic pump is used to produce an array containing various fractions of an unknown reactant solution. For example, an extract from a biological material such as leaf or a cell culture might contain various unknown materials, including receptors, ligands, alkaloids, nucleic acids, and even biological cells, some of which may have a desired activity. If a reservoir of such extract is electrophoretically pumped, the various species contained therein will move through the capillary at different rates. Of course, the various components being pumped should have some charge so that they can be separated. If the substrate is moved with respect to the dispenser while the extract components are being separated electrophoretically, an array containing various independent species is produced. This array is then tested for activity in a binding assay or other appropriate test. Those elements of the array that show promising activity are correlated with a fraction of the extract which is subsequently isolated from another source for further study. In some embodiments, the components in the extract solution are tagged with, for example, a fluorescent label. Then, during the process of delivering the solution with the electrophoretic pump, a fluorescence detector determines when labeled species are being deposited on the substrate. In some embodiments, the tag selectively binds to certain types of compounds within the extract, and imparts a charge to those compounds.

Other suitable delivery means include osmotic pumps and cell (biological) sorters. An osmotic pump delivers a steady flow of solution for a relatively long period. The construction of such pumps is well-known in the art, generally incorporating a solution of the extract of interest within a solvent permeable bag. Osmotic

pressure is applied to the extract solution by solvent molecules diffusing across the bag to equalize a concentration difference. The extract is thus forced out of a nozzle in the bag at a constant rate. Cell sorters are also well-known in the art, and can be used in applications where it is desirable to apply single biological cells to distinct locations on the substrate.

Although the above embodiments have been directed to systems employing liquid droplets, minuscule aliquots of each test substance can also be delivered to the cell as miniature pellets. Such pellets can be formed from the compound of interest (e.g. ligands for use in an affinity assay) and one or more kinds of inert binding material. The composition of such binders and methods for the preparation of the pellets will be apparent to those of skill in the art. Such "mini-pellets" will be compatible with a wide variety of test substances, stable for long periods of time, suitable for easy withdrawal from the storage vessel and dispensing (i.e., non-tacky, preferably suspendable in a liquid such as physiological buffer), and inert with respect to the binding activity of receptors.

In preferred embodiments, the reactant solutions in each predefined region are prevented from moving to adjacent regions by appropriate barriers or constraining regions. For example to confine aqueous monomer solutions, a hydrophilic material is used to coat the reaction regions, while a hydrophobic material is used in preferred embodiments to coat the region surrounding the individual reaction regions. Of course, when non-aqueous or nonpolar solvents are employed, different surface coatings are generally preferred. By choosing appropriate materials (substrates, hydrophobic coatings, and reactant solvents), the contact angle between the droplet and the substrate is advantageously controlled. Large contact angles between the reactant droplets and the substrate are desired because the solution then wets a relatively small reaction region with shallow contact angles, on the other hand, the droplet wets a larger area. In extreme cases, the droplet will spread to cover the entire surface.

The contact angle is determined by the following expression, known as Young's equation:

$$\cos\theta = (\sigma_{sa} - \sigma_{sl})/\sigma_{la}$$

where $\theta$ is the wetting angle, $\sigma_{sa}$ is the solid-air tension, $\sigma_{sl}$ is the solid-liquid tension, and $\sigma_{la}$ is the liquid-air surface tension. The values of these surface tensions are governed by thermodynamic considerations including the chemical constituents of the liquid and the solid substrate. The liquid-air surface tension for various

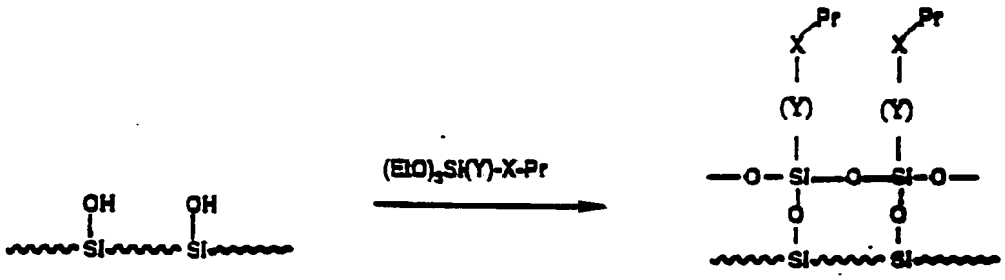
chemicals is easily measured by a variety of techniques such as those described in Adamson, Physical Chemistry of Surfaces, John Wiley and Sons, 5th Ed. (1990) which is incorporated herein by reference for all purposes. The difference of the solid-liquid and solid-air tensions can, for a given system, be determined empirically from a Zisman plot. In this approach, the contact angles are measured for a homologous series of liquids on a given solid surface. For some liquid in the series, a "critical contact angle" is observed, beyond which lower surface tension liquids wet the surface. The liquid-air surface tension of the liquid at this critical contact angle is assumed to be the surface tension of the solid. This approach has been found to provide quite reasonable results for low energy solids such as Teflon, polyethylene, hydrocarbons, etc. The information gained from such studies is used to optimize substrate compositions to increase wetting angles for given reactant solutions in the array.

Methods for controlling chemical composition and therefore the local surface free energy of a substrate surface include a variety of techniques apparent to those skilled in the art. Chemical vapor deposition and other techniques applied in the fabrication of integrated circuits can be applied to deposit highly uniform layers on selected regions of a surface. As a specific example, the wettability of a silicon wafer surface has been manipulated on the micrometer scale through a combination of self-assembled monolayer depositions and micromachining. See Abbott et al., "Manipulation of the Wettability of Surfaces on the .1 to 1 Micrometer Scale Through Micromachining and Molecular Self-Assembly" Science, 257 (Sept. 4, 1992) which is incorporated herein by reference for all purposes.

In a preferred embodiment, the perimeters of the individual regions are formed on a hydrophilic substrate defined by selectively removing hydrophobic protecting groups from the substrate surface. For example, a mono-layer of hydrophobic photoprotecting groups can be coupled to, for example, linker molecules attached to the substrate surface. The surface then is selectively irradiated (or otherwise activated by, for example, acid) through a mask to expose those areas where the reaction regions are to be located. This cleaves the protecting groups from the substrate surface, causing the reaction regions to be less hydrophobic than the surrounding area. This process produces a high density of reaction regions on the substrate surface. Because hydrophobic materials have lower surface free energies (surface tensions) than water, the solution droplet in the cell beads rather than spreads.

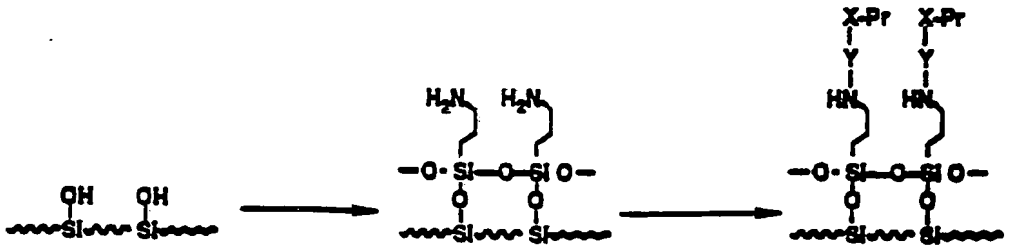
In some preferred embodiments, the substrate is prepared by first covalently attaching a monolayer of the desired reactive functional group (e.g. amine, hydroxyl, carboxyl, thio, etc.), which

is protected by a hydrophobic photolabile protecting moiety. If the substrate provides a glass surface, the monolayer may be deposited by a silanation reaction as shown below




In the above structures, Y is a spacer group such as a polymethylene chain, X is a reactive protected group such as NH, C(O)O, O, S, etc., and Pr is a hydrophobic photolabile protecting group.

In an alternative preferred embodiment shown below, the substrate surface is first derivatized by, for example, a silanation reaction with appropriates to provide an amine layer. A molecule including a spacer, a reactive group, and a photolabile group is then coupled to the surface.

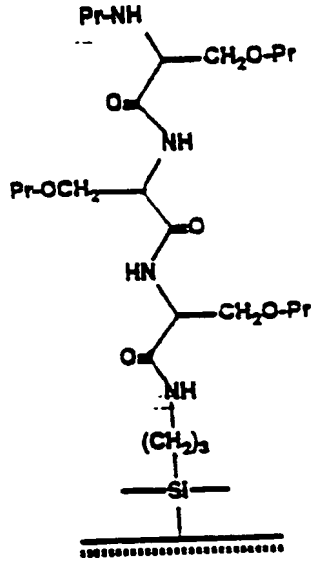



The photolabile protecting group should be sufficiently hydrophobic as to render the substrate surface substantially non-wettable. Removal of the protecting group in specific areas by exposure to light through a suitable mask, liberates the reactive functional groups. Because these groups are hydrophilic in character, they will render the substrate wettable in the exposed regions.

The class of nitrobenzyl protecting groups, which is exemplified by the nitroveratryl group, imparts significant

hydrophobicity to glass surfaces to which a member of the class is attached. The hydrophobicity of the basic nitrobenzyl protecting group is enhanced by appending group chain hydrocarbon substituent. Exemplary hydrophobic chains include $C_{12}H_{25}$ (lauryl) or $C_{18}H_{37}$ (stearyl) substituents. The syntheses of suitably activated forms (bromide, chloromethyl ether, and oxycarbonyl chloride) of a typical protecting group is schematically outlined in Fig. 14.

The spacer group ("Y") contributes to the net hydrophobic or hydrophilic nature of the surface. For example, those spacers consisting primarily of hydrocarbon chains, such as $-(CH_2)_n-$, tend to decrease wettability. Spacers including polyoxyethylene ($-(CH_2CH_2O)_n$), or polyamide ($-(CH_2CONH)_n$) chains tend to make the surface more hydrophilic. An even greater effect is achieved by using spacer groups which possess, in addition to the protected functional group, several "masked" hydrophilic moieties. This is illustrated below.




In preferred embodiments, the hydrophilic reaction regions is a two-dimensional circle or other shape having an aspect ratio near one (i.e. the length is not substantially larger or smaller than the width). However, in other embodiments, the hydrophilic region may take the form of a long channel which is used to direct flowing reactants in the manner described above.

In still other embodiments, the reaction regions are three-dimensional areas defined by, for example, gaskets or dimples on the substrate surface. The dimples or gaskets may also act as identification marks directing the dispenser to the region of interest.

If the solvent (or other liquid used to deliver the reactant) has a sufficiently high vapor pressure, evaporation can cause the reactant concentration to increase. If left unchecked, this process ultimately causes the solute to precipitate from solution. The effects of evaporation can be minimized by sealing selected regions of the substrate when they need not be accessible. Alternatively, the partial pressure of volatile reagents can be adjusted so that the liquid and vapor phase fugacities are equalized and the thermodynamic force driving evaporation is reduced. The partial pressure of the reagents may be increased by providing a relatively large reservoir of volatile reagents in a sealed chamber. For example, solvents having a low vapor pressure under the conditions of interest can be used. In some cases, evaporation can be further controlled by application of a film or coverplate having a reverse array pattern. Other methods of preventing evaporation are well-known in the physical chemical arts and may be used in the present invention.

In some preferred embodiments, evaporation is advantageously employed to accelerate hybridization of target oligonucleotides with immobilized oligonucleotides in the reaction regions. In one specific embodiment, fluorescently tagged or otherwise labelled target oligonucleotides in solution (e.g., a solution containing a salt such as ammonium acetate or magnesium chloride) are delivered to reaction regions containing immobilized probe oligonucleotides. As the volatile salt solution evaporates from the reactant droplet (in the same manner as solvent evaporates from an ink droplet deposited by an ink jet printer), a locally high concentration ratio of target to probe oligonucleotide results, accelerating hybridization. If hybridization is carried out at room temperature, ten minutes to a few hours are typically required to complete the reaction. After sufficient time, the unhybridized DNA is washed or otherwise removed from the substrate. Finally, the substrate is imaged to detect regions in which the probe and target DNA have hybridized. Of course, evaporation can be advantageously employed to increase the local concentration of non-DNA solutes in a variety of reactions besides hybridization. For example in some embodiments, receptor solutions are sufficiently volatile that the local receptor concentration increases in the reaction regions containing peptides, for example, to be screened.

The arrays produced according to the above spotting embodiments are generally used in much the same manner as the arrays produced by the flow channel embodiments described above. For example, the arrays can be used in screening with fluorescein labelled receptors as described in PCT Publication No. WO92/10092, previously incorporated by reference.

VI. Alternative Embodiments

According to some embodiments of the invention, microvalve structures are used to form channels along selected flow paths on the substrate. According to these embodiments, an array of microvalves is formed and operated by an overlying or underlying array of electrodes that is used to energize selected valves to open and close such valves.

Fig. 15 illustrates such a structure, Fig. 15a illustrating the system in end view cross-section and Fig. 15b illustrating the system in top view. The structure shown therein provides for only two synthesis chambers for the purpose of clarity, but in most embodiments a far greater number of chambers will be provided. Microvalves are discussed in detail in, for example, Zdeblick, U.S. Patent No. 4,966,646, and Knutti, "Advanced Silicon Microstructures," ASICT Conference (1989), both incorporated herein by reference for all purposes.

As shown therein, a substrate 602 is provided with a plurality of channels 604 formed using photolithographic, or other related techniques. The channels lead up to a synthesis chamber 606. At the end of each channel is valve structure 608. As shown in Fig. 15, the channels lead up to the chambers, but may be isolated from the chambers by the valves. Multiple valves may be provided for each chamber. In the particular structure shown in Fig. 15, the right valve on the left chamber and the left valve on the right chamber are open while the remaining valves are closed. Accordingly, if reagent is delivered to the top of the substrate, it will flow through the open channel to and through the chamber on the left, but not the one on the right. Accordingly, coupling steps may be conducted on the chamber with selected reagents directed to selected chambers, using the techniques discussed above.

According to some embodiments, a valve is supplied on one side of the chamber 606, but the valve on the opposite side is replaced by a semi-permeable membrane. According to these embodiments, it becomes possible to flow a selected reagent into the chamber 606 and, thereafter, flow another selected reagent through the flow channel adjacent the semi-permeable membrane. The semi-permeable membrane will permit a portion of the material on one side

or the other to pass through the membrane. Such embodiments will be useful in, for example, cell studies.

Screening will be performed by, for example, separating or cutting two halves of the device, enabling screening by, for example, contacting with a fluorescein labelled antibody, or the like followed by photodetection.

Figs. 16a and 16b illustrate another alternative embodiment of the invention which combines the mechanical polymer synthesis techniques disclosed herein with light-directed synthesis techniques. According to these embodiments, a substrate 401 is irradiated in selected regions, shown as the stripes in Fig. 16a. The surface of the substrate is provided with photoremovable groups in accordance with PCT Publication No. WO92/10092 (previously incorporated by reference) on, for example, amine groups in the specific case of peptide synthesis. During this step regions 701, 702, and 703 of the substrate, among others, are deprotected, leaving remaining regions of the substrate protected by photoremovable groups such as nitroveratryl oxycarbonyl ("NVOC"). According to a specific embodiment of the invention the widths of the irradiated regions equal the widths of the protected regions of the substrate.

Thereafter, as shown in Fig. 16b the substrate is contacted with a channel block 407. In the particular embodiment shown in Fig. 16b, the channels 704, 705, and 707 are aligned with the regions 701, 702, and 703, respectively, on the substrate 401. As will be apparent, specific embodiments of the invention provide for irradiated regions and channels in the form of stripes, which are aligned during this step. Other embodiments, however, will provide for other shapes of irradiated regions and channels, and other relative orientations of the irradiated regions and channels. The channel block and substrate will be aligned with, for example, an alignment mark placed on both the substrate and the channel block. The substrate may be placed on the channel block with, for example, a vacuum tip.

Thereafter, a selected reagent is flowed through or placed in the channels in the channel block for coupling to the regions which have previously been exposed to light. As with the flow channel embodiments described above, the substrate may be placed in contact with a prefilled channel block in some embodiments to avoid compression of the channel block to the substrate and dead spots during pumping. According to preferred aspects of the invention, a different reagent flows through each of the channels 701, 702, and 703 such as, for example, a reagent containing monomers A, B, and C. The process may then, optionally, involve a second coupling step in which the substrate is translated by, e.g., one

channel width, to provide coupling of a monomer in the regions between the original channels.

Thereafter, the process of directed irradiation by light, followed by coupling with the channel block is repeated at the previously unexposed regions. The process is then preferably repeated again, with the stripes of the mask and the channel block rotated at, for example, 90 degrees. The coupling steps will provide for the formation of polymers having diverse monomer sequences at selected regions of the substrate through appropriate translation of the mask and substrate, and through appropriate mask selection. Through a combination of the light-directed techniques and the mechanical flow channel techniques disclosed herein, greater efficiency in forming diverse sequences is achieved, because multiple monomers are coupled in a single irradiation/coupling step.

In light-directed methods, the light shown through the mask is diffracted to varying degrees around the edges of the dark regions of the mask. Thus, some undesired removal of photosensitive protecting groups at the edges of "dark" regions occurs. This effect is exacerbated by the repeated mask translations and subsequent exposures, ultimately leading to inhomogeneous synthesis sites at the edges of the predefined regions. The effect is, of course, dependent upon the thickness of the glass substrate and the angle at which the light is diffracted. If the mask is positioned on the "backside" of the substrate, a diffraction angle of 2.5° and a substrate thickness of 0.7 mm creates a 60 μm strip of light (of variable intensity) flanking each edge. For a 0.1 mm thick substrate, the strip is approximately 5 μm wide.

To reduce these "bleed-over" effects of diffraction, a pinhole mask may be employed to activate and/or define reaction regions of the substrate. Thus, for example, light shown through the pinhole mask is directed onto a substrate containing photoremovable hydrophobic groups. The groups in the illuminated regions are then removed to define hydrophilic reaction regions. In one specific embodiment, the pinhole mask contains a series of circular holes of defined diameter and separation, e.g., 20 μm diameter holes spaced 50 μm apart. In some preferred embodiments, a stationary pinhole mask is sandwiched between the substrate and a translational mask of the type described in PCT Publication No. WO92/10092. In this manner selected regions of the substrate can be activated for polymer synthesis without bleed-over. The translational mask is used to illuminate selected holes of the stationary pinhole mask, and is aligned such that its edges dissect the distance separating the holes of the stationary mask thereby eliminating diffractive removal of photoprotecting groups at neighboring sites. Because there is negligible bleed-over incident light, inhomogeneous synthesis at

sites juxtaposed along the edge is eliminated. The resulting circular sites do, of course, contain variable sequence density due to diffraction at the edges of the pinhole mask, but the sequences at each predefined region are homogeneous. In addition, each synthesis region is surrounded by a "dark" region when the substrate is probed with a labeled target. Thus, no bleed-over fluorescence signal is introduced by binding at neighboring sites.

A pinhole mask containing 20 μm circular holes separated by 50 μm requires a total synthesis area for the complete set of octanucleotides of only 1.78 $cm^2$. For a given pinhole mask, thinner substrates allow for smaller reaction sites separated by larger distances. However, the area from which reliable data can be obtained is also reduced when smaller sites are used. The density of reaction sites is ultimately determined by the diffraction angle and the distance between the pinhole mask and the reaction regions (typically the substrate thickness).

Although the discussion so far has focused upon circular pinholes, other shapes such as slots, squares, crescents, etc. may be employed as is appropriate for the selected delivery method. Thus, for some flow channel embodiments, linear or serpentine slots may be desired.

In alternative preferred embodiments, the pinhole mask takes the form of a layer coated on the substrate. This avoids the need for a separate stationary mask to generate the dot pattern. In addition, the surface layer provides well defined synthesis regions in which to deposit reactants according to the spotting embodiments described above. Further, the surface pinhole mask is conveniently embossed with local reference coordinates for use in navigational systems used to deliver monomer solutions to proper regions as described above. Preferred pinhole masks are made from opaque or reflective materials such as chrome.

## VI. Examples

### A. Leak Testing

An initial experiment was conducted using a flow channel device to ensure that solutions could be delivered to selected locations of a substrate and be prevented from contacting other areas. Additionally, the experiment was used to demonstrate that reagents could be delivered in a uniform manner.

Accordingly, a flat piece of conventional glass having dimensions of about 42 mm x 42 mm was derivatized with aminopropyltriethoxysilane. The entire slide was deprotected and washed using conventional techniques. A fluorescein marker of FITC was then injected into flow channels formed when a block of KelF™ 81 with 10 channels of 1 mm depth and 1 mm width were brought into

contact with the substrate. The fluorescein marker was in a solution of DMF and flowed through the channels by injecting the material into the groove with a manual pipet.

Fluorescein dye was similarly injected into every other channel in the block, the block was rotated, and the process was repeated. The expected resulting plot of fluorescent intensity versus location is schematically illustrated in Fig. 17. Dark regions are shown at the intersections of the vertical and horizontal stripes, while lighter grey at non-intersecting regions of the stripes. The dark grey regions indicate expected regions of high dye concentration, while the light regions indicate regions of expected lower dye concentration.

A mapping was made of fluorescence intensity of a portion of an actual slide, with intensity data gathered according to the methods of PCT Publication No. WO92/10092, previously incorporated by reference. The results agree closely with the expected results, exhibiting high fluorescence intensity at the intersection of the channels (about 50% higher than non-intersecting regions of the stripes), and lower fluorescence intensity at other regions of the channels. Regions which were not exposed to fluorescence dye show little activity, indicating a good signal-to-noise ratio. Intersections have fluorescence intensity about 9x as high as background. Also, regions within the channels show low variation in fluorescence intensity, indicating that the regions are being evenly treated within the channels.

B. Formation of YGGFL

The system was used to synthesize four distinct peptides: YGGFL (SEQ. ID NO:1), YpGFL (SEQ. ID NO:2), pGGFL (SEQ. ID NO:3), and ppGFL (the abbreviations are included in Stryer, Biochemistry, Third Ed. (1988), previously incorporated herein by reference; lower case letters indicate D-optical isomers and upper case letters indicate L-optical isomers). An entire glass substrate was derivatized with TBOC-protected aminopropyltriethoxysilane, deprotected with TFA, coated with FMOC-protected caproic acid (a linker), deprotected with piperidine, and coated with FMOC-protected Glycine-Phenylalanine-Leucine (GFL).

This FMOC-GFL-coated slide was sealed to the channel block, and all 10 grooves were deprotected with piperidine in DMF. After washing the grooves, FMOC Glycine (G) was injected in the odd grooves, and FMOC d-Proline (p) was injected in the even grooves. After a two-hour coupling time, using standard coupling chemistry, all grooves were washed with DMF. The grooves were vacuum dried, the block removed and rotated 90 degrees. After resealing, all grooves were deprotected with piperidine in DMF and washed. FMOC Tyrosine

(Y) was injected in the odd grooves, and FMOC p in the even grooves. After coupling the grooves were washed and vacuum dried. Accordingly, 25 regions of each of the compounds YGGFL, YpGFL, pGGFL, and ppGFL were synthesized on the substrate. The substrate was removed and stained with FITC-labelled antibodies (Herz antibody 3E7).

The resulting slide showed bright regions of high fluorescence. White squares are in locations of YGGFL. The darkest regions are pGGFL and ppGFL. The YGGFL sites were the most intense, followed by the YpGFL sites. The pGGFL and ppGFL intensities were near background levels, consistent with expected results with the Herz antibody.

Quantitative analysis of the results show overall intensity ratios for YGGFL:YpGFL:pGGFL:ppGFL as 1.7:1.5:1.1:1.0. However, since there is a large standard deviation on the YGGFL and YpGFL, comparing all the sites with each other may not accurately represent the actual contrasts. Comparing the intensities of sites within the same "stripe" gives larger contrasts, although they remain on the order of 2:1.

C. 100 Micron Channel Block

A grid pattern of fluorescein isothiocyanate coupled to a substrate was made by using a flow cell of this invention. A two by three inch NVOC-derivatized substrate was photolyzed through a mask to produce 400 micron activated bands on one axis. An etched silicon channel block having 64 parallel 100 micron channels separated by 100 micron walls was then clamped to the substrate on the other axis (i.e., perpendicular to the axis of 400 micron activated bands). The clamping assembly consisting of aluminum top and bottom clamp plates was used. Pressure was applied by tightening two bolts with a torque wrench to 400 psi. A 7 mM fluorescein isothiocyanate solution was flowed through the channels by pipetting directly to exposed channel ends.

An image of the substrate showed regions of high fluorescence indicating that the fluorescein had bound to the substrate. White squares indicating fluorescein binding were present as 400 micron horizontal stripes on the photolyzed regions within the 100 micron vertical flow paths. Contrast ratios of 8:1 were observed between the channels and the channel spacings. This demonstrates the nearly complete physical isolation of fluid passing through 100 micron channels under 400 psi of clamping pressure.

D. Channel Matrix Hybridization Assay

A center region of a two by three inch slide was derivatized with bis(2-Hydroxyethyl) aminopropyltriethoxy silane.

Six nucleosides were then coupled to the entire reaction region using a synthesis process consisting of deprotection, coupling, and oxidation steps for each monomer applied. These first six nucleosides were coupled in a reaction region defined by a 0.84 inch diameter circular well in an aluminum template clamped to the two by three inch slide.

The seventh and eighth monomers were applied to the substrate by flowing monomer solutions through 100 micron channels in an etched silicon channel block (employed in Example C above). The seventh base was coupled along the long axis (vertical) of the two-inch by three-inch slide, and the eighth base perpendicular to the seventh, along the short axis (horizontal) of the slide. This defined an active matrix region of 1.28 by 1.28 cm having a density of 2,500 reaction regions per square centimeter.

The channel block was centered over the reaction region and clamped to the substrate using a clamping assembly consisting of machined aluminum plates. This aligned the two inch by three inch substrate relative to the channel block in the desired orientation. Rotation of the top clamp plate and channel block relative to the bottom clamp plate and substrate between the seventh and eighth coupling steps provided for the matrix of intersecting rows and columns.

In the top clamp plate, fluid delivery wells were connected to laser-drilled holes which entered individual channels from the back surface of the channel block. These delivery wells were used to pipette coupling reagents into channels while the channel block was clamped to the substrate. Corresponding fluid-retrieval wells were connected to vacuum at the downstream of the channel block, drawing fluid through the channels and out to a waste container. Thus continuous fluid flow over the substrate in the channel region during coupling steps was achieved.

The complete octamer synthesized at the channel intersections formed by the seventh and eighth coupling steps had the following sequence:

Substrate--(3')CGCAGCCG(5') (SEQ. ID NO:4).

After completion of the synthesis process, cleavage of exocyclic amines was performed by immersion of the reaction region in concentrated ammonium hydroxide. The reaction region was then incubated at 15°C for one hour in a 10 nM solution of the complementary base sequence 5' GCGTCGGC-F (SEQ. ID No:5), where "F" is a fluorescein molecule coupled to the 3' end of the oligonucleotide. The target chain solution was then flushed from the reaction region and replaced with neat 6x SSPE buffer, also at 15°C. Finally, the reaction region was then scanned using a laser fluorescence detection system while immersed in the buffer.

The brightest regions in the resulting image correspond to channel intersections where a full octamer was synthesized on the substrate surface. Vertical columns on the image displayed the channel regions where the seventh base was coupled, while horizontal rows display the channel regions where the eighth base was coupled. Brightness in the channel intersection regions indicated hybridization between the fluoresceinated target chain and the complementary chain synthesized and bound to the substrate in these regions. The vertical stripes of the image showed a consistent brightness with regions of significantly greater brightness at the intersection regions. The horizontal stripes did not contain the consistent brightness of the vertical stripes, but did have regions of brightness at the intersections with the vertical stripes. The consistent brightness along the seventh monomer axis (vertical) indicated partial hybridization of the target chain in areas where seven of the eight complementary bases were coupled to the substrate surface. The lack of brightness along the eighth monomer axis (horizontal) is consistent with the expectation that a chain of six matching bases bound to the substrate surface will not hybridize effectively to an octamer in solution (heptamers with six matching bases followed by a mismatch at the seventh position). The darker background consists of hexamers consisting of the first six monomers coupled to the entire reaction region.

VII. Conclusion

The above description is illustrative and not restrictive. Many variations of the invention will become apparent to those of skill in the art upon review of this disclosure. Merely by way of example a variety of substrates, receptors, ligands, and other materials may be used without departing from the scope of the invention. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Combinatorial Strategies For Polymer Synthesis

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Gly Gly Phe Leu
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Gly Phe Leu
1

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Gly Phe Leu
1

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCGACGC

8

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGTCGGC
8

WHAT IS CLAIMED IS:

1. A method of forming polymers having diverse monomer sequences on a single substrate, said substrate comprising a surface with a plurality of selected regions, said method comprising the steps of:

a) forming a plurality of channels adjacent said surface, said channels at least partially having a wall thereof defined by a portion of said selected regions;

b) placing selected monomers in said channels to synthesize polymers at said portion of said selected regions, said portion of said selected regions comprising polymers with a sequence of monomers different from polymers in at least one other of said selected regions; and

c) repeating steps a) and b) with said channels formed along a second portion of said selected regions.

2. The method as recited in claim 1 wherein said step of forming a plurality of channels comprises the step of placing a channel block adjacent said surface, said channel block having a plurality of grooves therein, walls of said grooves and said surface at least partially defining said flow channels.

3. The method as recited in claim 1 wherein the step of placing selected reagents in said channels comprises the steps of:

removing a protecting group from an active site in at least a first channel;

flowing a first monomer through said at least a first channel, said first monomer comprising a protecting group thereon, said first monomer binding to said active site in said first channel;

removing a protecting group from said active site in at least a second channel, at least a portion of said second channel overlapping a portion of said substrate contacted by said first channel; and

flowing a second monomer through said at least a second channel, said second monomer binding to said active site in said second channel.

4. The method as recited in claim 1 further comprising the step of screening said polymers for binding affinity with a receptor.

5. The method as recited in claim 1 wherein at least 10 different polymers are formed on said surface.

6. The method as recited in claim 1 wherein at least 1,000 different polymers are formed on said surface.

7. The method as recited in claim 1 wherein at least 100,000 different polymers are formed on said surface.

8. The method as recited in claim 1 wherein said polymers are selected from the group consisting of oligonucleotides and peptides.

9. The method as recited in claim 1 wherein said selected regions each have an area of less than about 10,000 microns$^2$.

10. The method as recited in claim 3 wherein the steps of removing and flowing further comprise the steps of:

placing a channel block in contact with said surface in a first orientation and placing a material comprising a first monomer through at least one channel in said channel block;

rotating one of said channel block and said substrate relative to the other; and

placing a channel block in contact with said surface in a second orientation and placing a material comprising a second monomer through at least one channel in said channel block.

11. The method as recited in claim 1 wherein said steps of placing selected reagents in said channels comprises:

placing a pipet in fluid communication with said channel; and

injecting said selected reagents through said channels.

12. The method as recited in claim 11 wherein said step of placing a pipet in fluid communication with said channel is a step of placing said pipet in contact with an orifice on a side of said substrate opposite said surface.

13. The method as recited in claim 11 wherein said step of placing a pipet in fluid communication with said channel is a step of placing a plurality of pipets in communication with a plurality of said channels and flowing different reagents through at least two of said channels.

14. The method as recited in claim 1 preceded by the step of forming an array of valves of said surface whereby fluid may be directed to desired locations on said surface, and selectively

operating said valves and flowing said selected reagents through channels formed thereby.

15. The method as recited in claim 1 preceded by the step of irradiating portions of said substrate with light whereby photoremovable groups are removed from active groups on said substrate.

16. The method as recited in claim 15 wherein said selected irradiated portions are in the form of stripes, and wherein said step of forming channels comprises forming said channels along a path of said stripes, different reagents placed in at least a portion of said channels.

17. A method of forming a plurality of peptide sequences on a surface of a single substrate comprising the steps of:

a) placing said substrate in contact with a channel block in a first orientation, said channel block having a plurality of channels therein;

b) flowing at least a first amino acid through at least one of said channels, coupling said first to portions of said surface;

c) flowing at least a second amino acid through at least one of said channels, coupling said second amino acid to portions of said surface;

d) rotating said channel block relative to said substrate and placing said substrate in contact with said channel block again;

e) flowing a third amino acid through at least one of said channels to form at least first and second peptide sequences on said surface; and

f) flowing a fourth amino acid through at least one of said channels to form at least third and fourth peptide sequences on said surface.

18. A kit for forming diverse polymer sequences comprising:

a substrate;

a channel block, said channel block having a plurality of grooves therein;

means for holding said channel block in engagement with said substrate;

means for translating said channel block and said substrate relative to the other; and

means for injecting selected reagents into said grooves.

19. The kit as recited in claim 18 wherein said substrate comprises active site protecting groups.

20. The kit as recited in claim 19 further comprising a deprotecting material for removal of said protecting groups.

21. The kit as recited in claim 18 wherein said grooves are connected to apertures in said channel block, said apertures extending through a back surface of said substrate.

22. The kit as recited in claim 18 further comprising means for irradiating selected portions of said substrate for removal of protecting groups on said substrate, said protecting groups removed from active sites on said substrate upon exposure to light.

23. The kit as recited in claim 22 wherein said means for irradiating comprises a light source and a light mask, said light mask comprising regions opaque to said light and regions transmissive to said light.

24. The kit as recited in claim 18 wherein said means for injecting comprises a pipettor.

25. The kit as recited in claim 24 wherein said pipettor comprises a plurality of pipettes, each of which is coupled to a different one of said grooves.

26. A system for conducting a plurality of reactions on a single substrate, the system comprising:

at least about 100 reaction regions on the single substrate, each reaction region being capable of conducting a separate reaction;

means for delivering one or more reactants to one or more of the reaction regions; and

means for constraining at least some of the reactants from contacting at least some of the reaction regions.

27. The system recited in claim 26 wherein the substrate comprises a plurality of passages and the reactions are supports within said passages.

28. The system recited in claim 26 wherein the means for delivering the one or more reactants are flow channels of a channel block adjacent the substrate and the means for constraining at least some of the reactants are walls of the flow channel.

29. The system recited in claim 26 wherein the means for constraining at least some of the reactants is a hydrophobic layer on the surface of the substrate.

30. A substrate comprising

greater than about 100 reaction regions having different compounds therein;

a constraining region surrounding the reaction regions, the constraining region being more hydrophobic than the reaction regions.

31. The substrate recited in claim 30, wherein the constraining region comprises hydrophobic protecting groups.

32. The substrate recited in claim 31 wherein the protecting groups are photolabile.

33. The substrate recited in claim 30 wherein the reaction regions define channels.

34. The substrate recited in claim 30 wherein the substrate includes greater than about 1000 reaction regions.

35. A method of forming a plurality of polymers having diverse monomer sequences on a substrate, the substrate including a plurality of reaction regions surrounded by a constraining region, the reaction regions being more wettable by one or more monomer solutions than the constraining region, the method comprising

sequentially placing the one more monomer solutions in a first reaction region to form a first polymer having a first monomer sequence, the monomer solutions being confined to the first reaction region by the constraining region; and

sequentially placing the one more monomer solutions in a second reaction region to form a second polymer having a second monomer sequence, the monomer solutions being confined to the first reaction region by the constraining region.

36. The method recited in claim 35 wherein the steps of placing monomer solutions in the first reaction region include moving a pipette with respect to the substrate and depositing at least one monomer solution in the first reaction region.

37. The method recited in claim 35 further comprising steps of periodically removing monomer solutions from the first and second reaction regions after selected monomers have been coupled to the first and second polymers.

38. The method recited in claim 35 wherein a first monomer is coupled in the first reaction region and a second monomer is coupled in the second reaction regions before additional monomers are placed and coupled in the first and second reaction regions.

39. The method recited in claim 35 wherein the monomer solutions are placed in the first and second reaction regions by a dispenser selected from the group consisting of an electrophoretic pump, a pipette, and a charged drop dispenser.

40. A method of transforming a first heterogeneous array of compounds on a single substrate, the heterogeneous array of compounds having a plurality of reaction regions, the method comprising the following steps:

activating a first group of reaction regions and a second group of reaction regions;

delivering a first reactant to the first group of reaction regions but not to the second group of reaction regions;

allowing the first reactant to react at the first group of reaction regions, to convert the first heterogeneous array

into a second heterogeneous array, wherein the heterogeneous arrays have greater than about 100 distinct reaction regions.

41. The method recited in claim 40 further comprising a step of isolating the first group of reaction regions from the second group of reaction regions.

42. The method recited in claim 41 wherein the first group of reaction regions is isolated by placing a channel block against the substrate.

43. The method recited in claim 40 wherein the first group of reaction regions is isolated from the second group of reaction regions by walls on the substrate.

44. The method recited in claim 43 wherein the substrate includes a series of flow through reaction regions separated from one another by walls.

45. The method recited in claim 40 wherein the first group of reaction regions is isolated from the second group of reaction regions by one or more non-wetting regions on the substrate.

46. The method recited in claim 40 wherein the heterogeneous arrays have greater than about 1000 distinct reaction regions.

47. The method recited in claim 40 further comprising the following steps:

delivering a second reactant to the second group of reaction regions but not to the first group of reaction regions;

allowing the second reactant to react at the second group of reaction regions;

activating a third group of reaction regions, the third group having some reaction regions in common with the first group of reaction regions;

delivering a reactant to the third group or reaction regions but not to the second group of reaction regions; and

allowing the a reaction to take place in the third group of reaction regions.

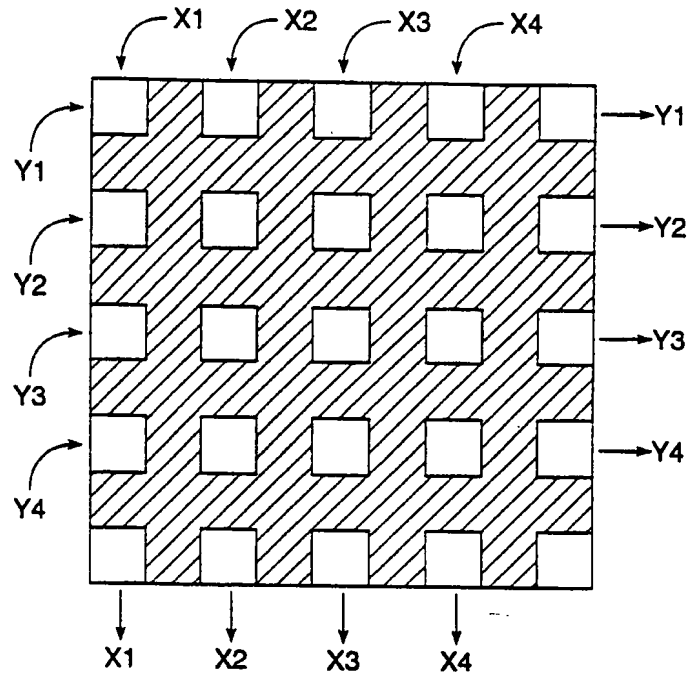

X1
X2
X3
X4
Y1
Y2
Y3
Y4
Y1
Y2
Y3
Y4
X1
X2
X3
X4

FIG. 1

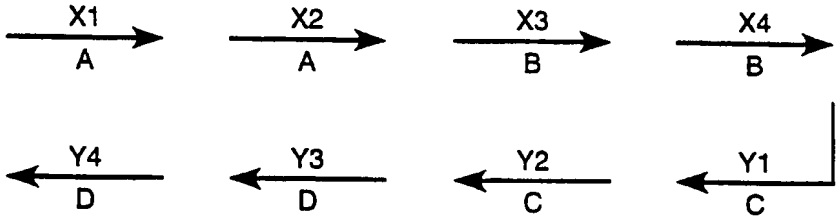
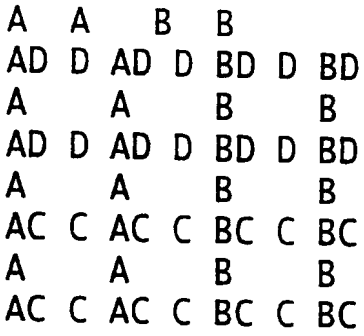

X1
A
X2
A
X3
B
X4
B
Y4
D
Y3
D
Y2
C
Y1
C

FIG. 2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A A B B
AD D AD D BD D BD
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AC C AC C BC C BC
A A B B
AC C AC C BC C BC

*FIG. 3*

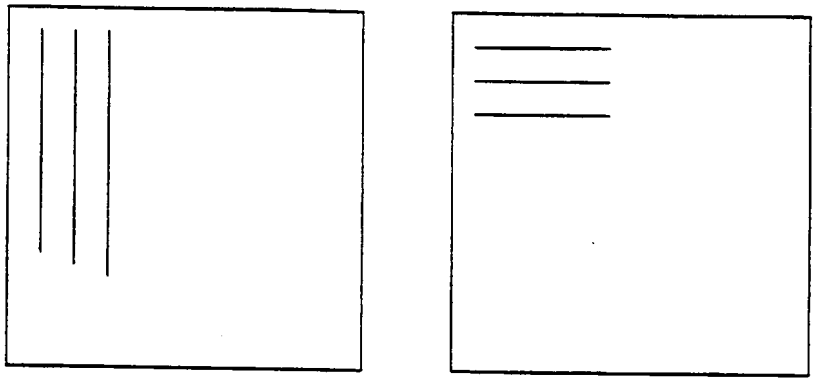

FIG. 4A

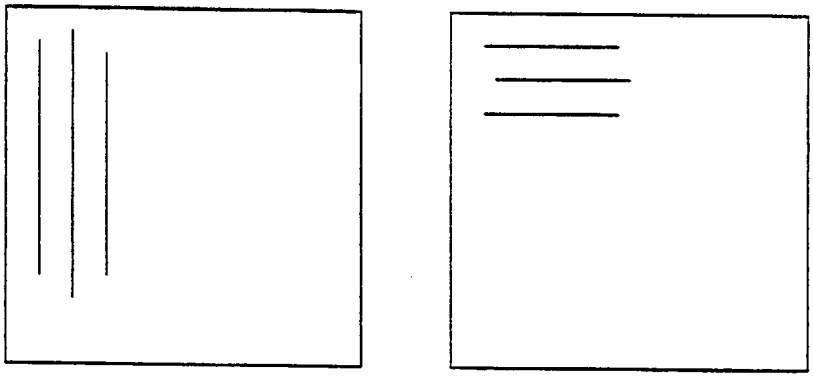

FIG. 4B

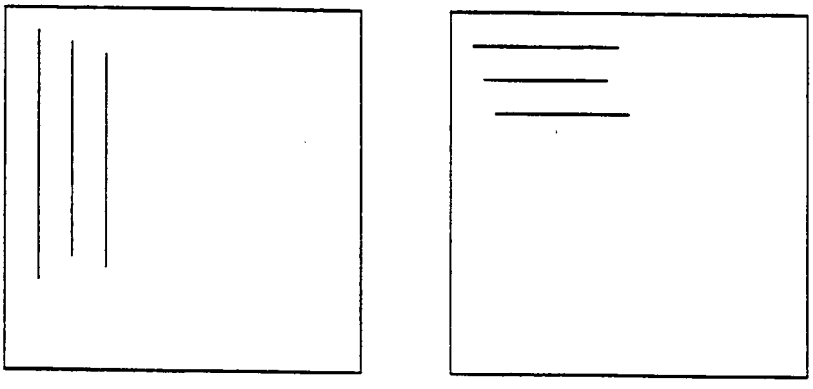

FIG. 4C

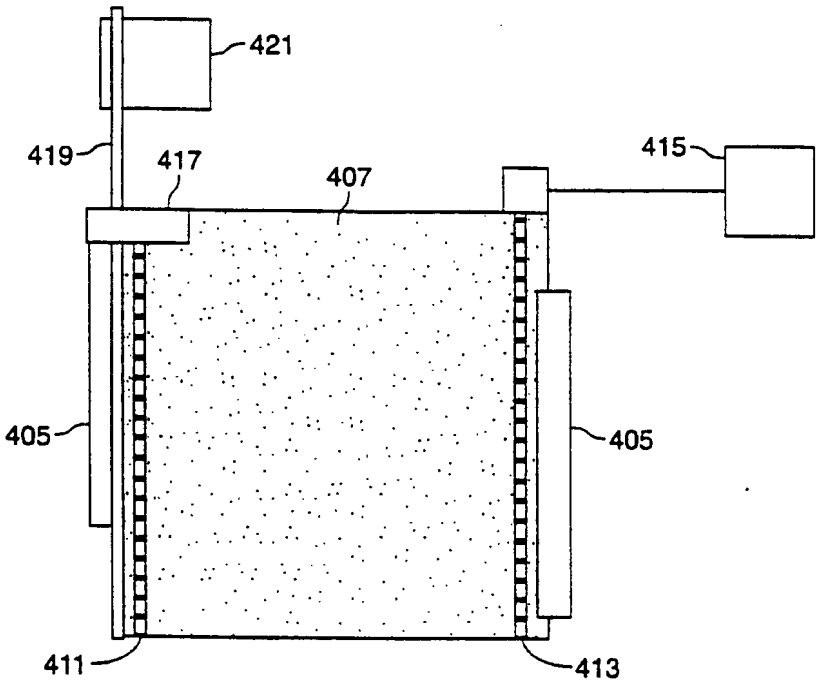
421
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FIG. 5A

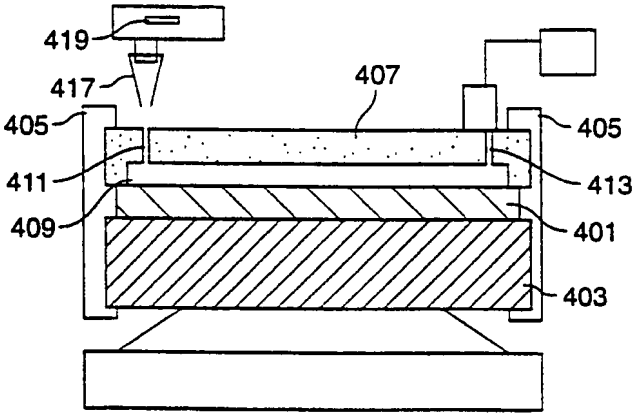
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FIG. 5B

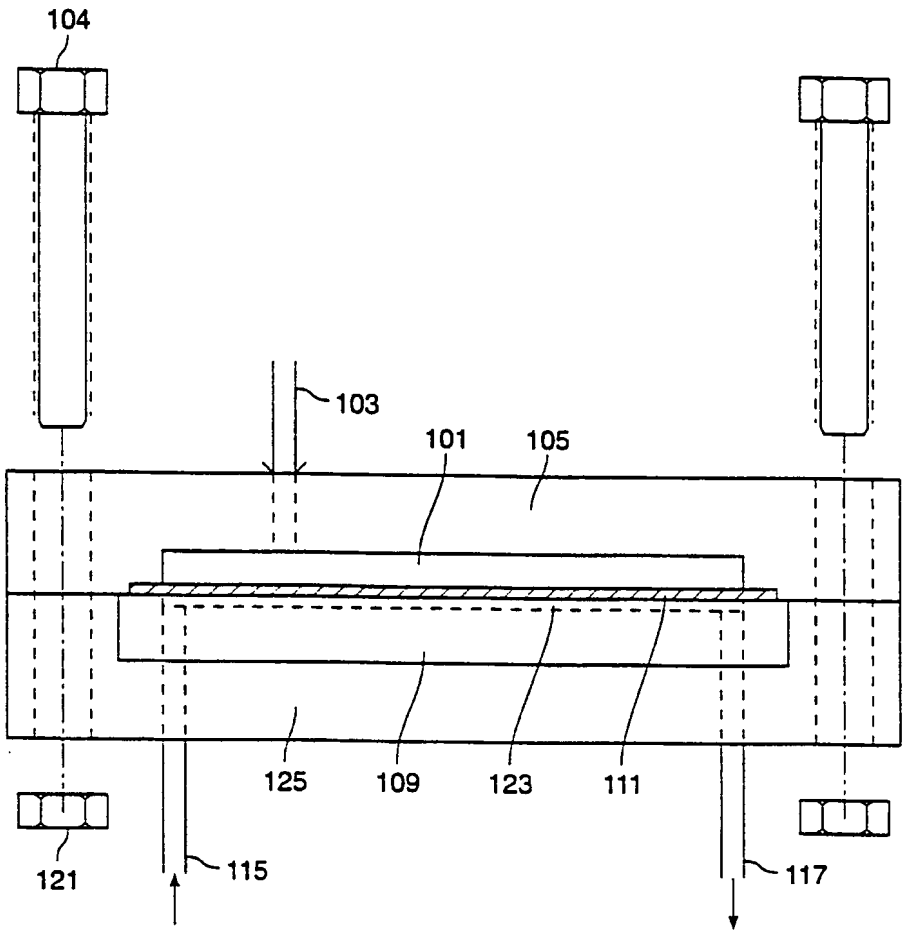



FIG. 6

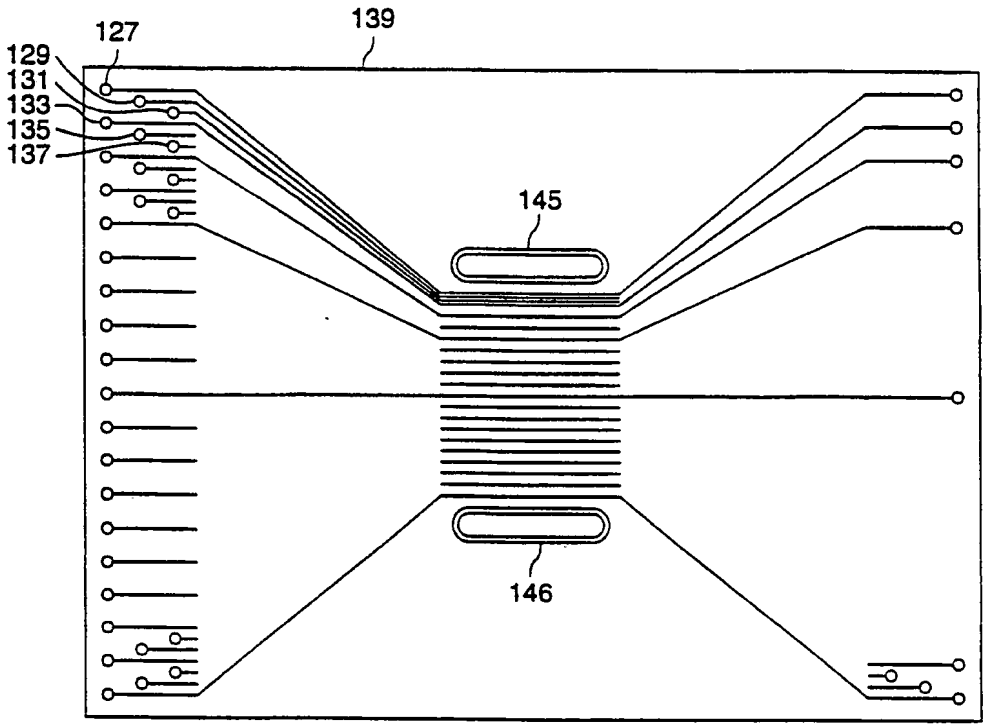



FIG. 7A

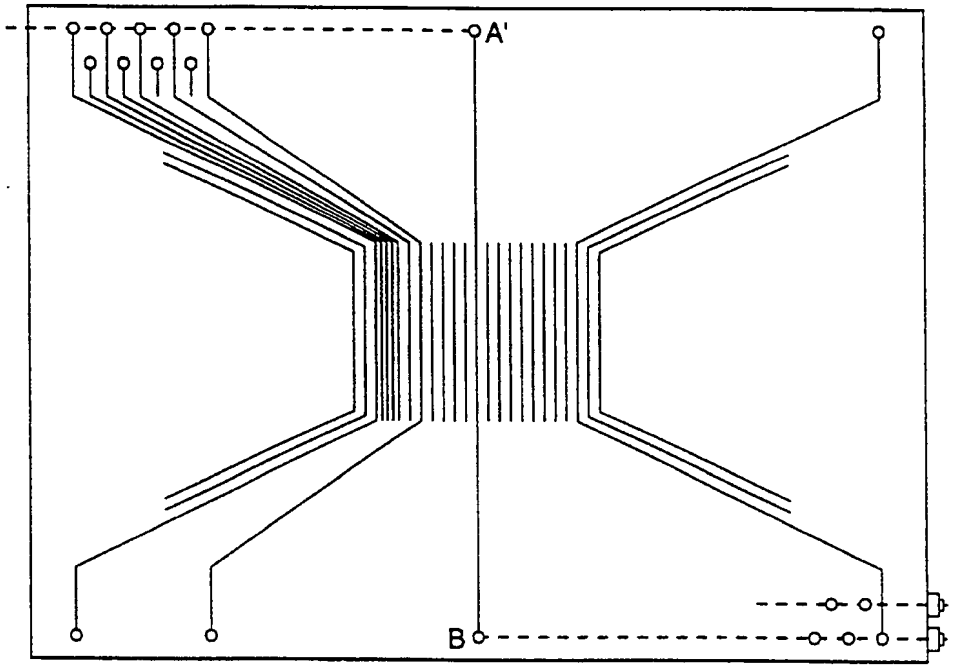



FIG. 7B

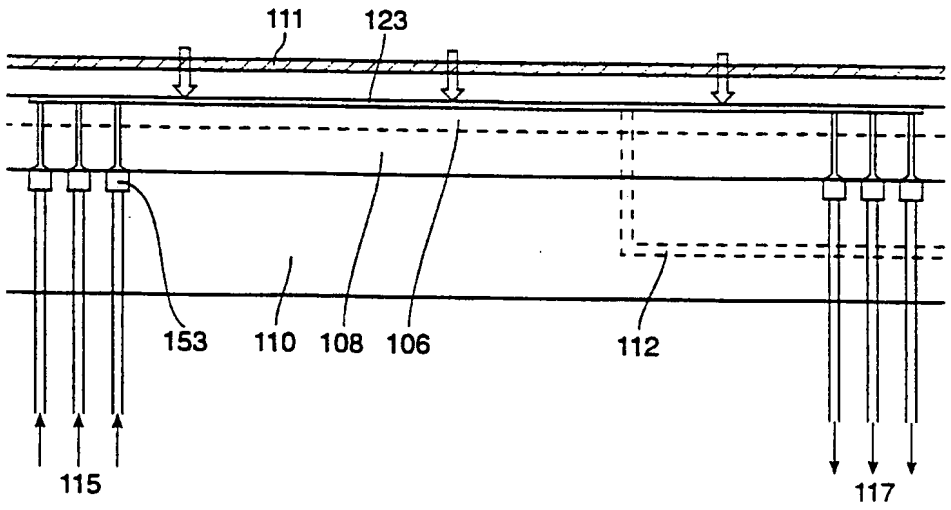



FIG. 8

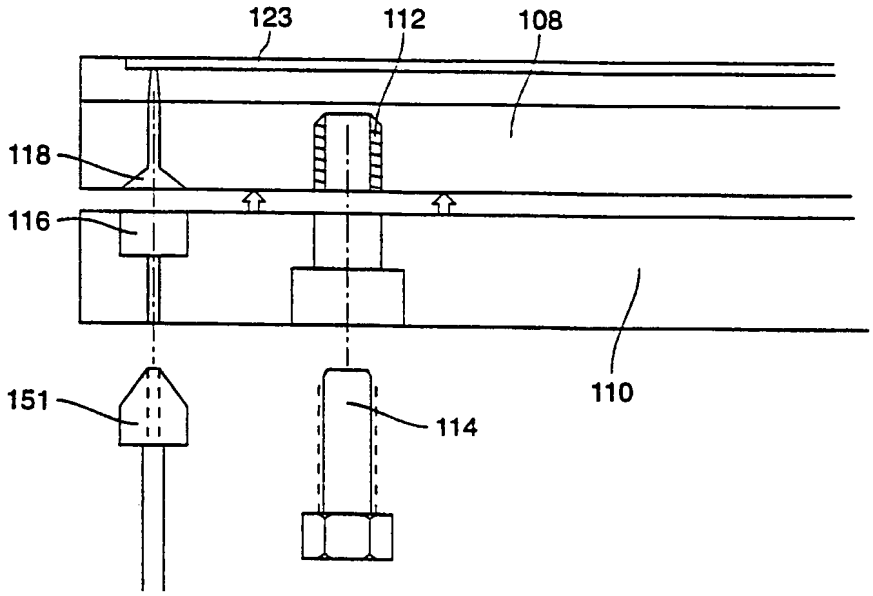



*FIG. 9*

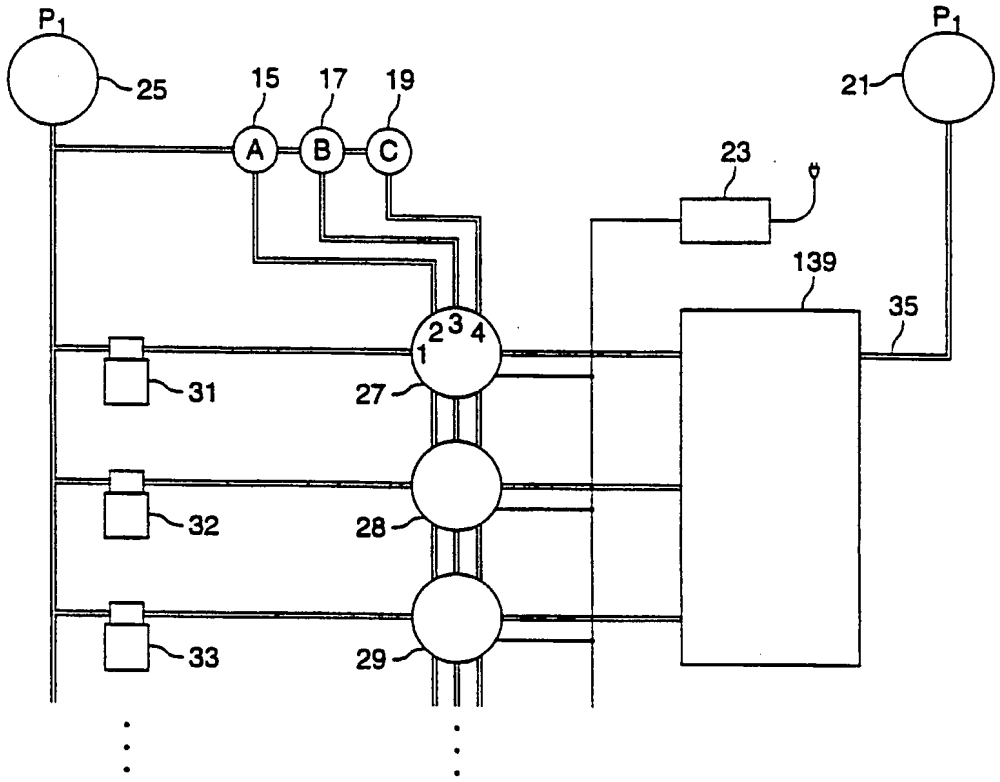



FIG. 10

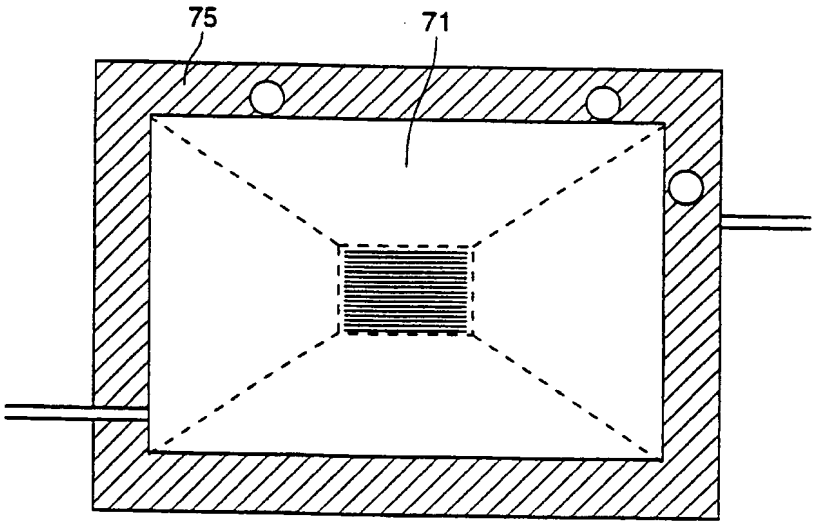



FIG. 11A

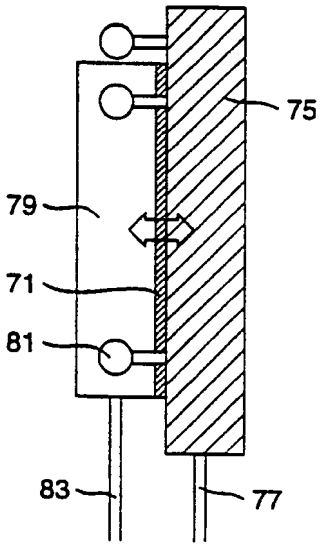



FIG. 11B

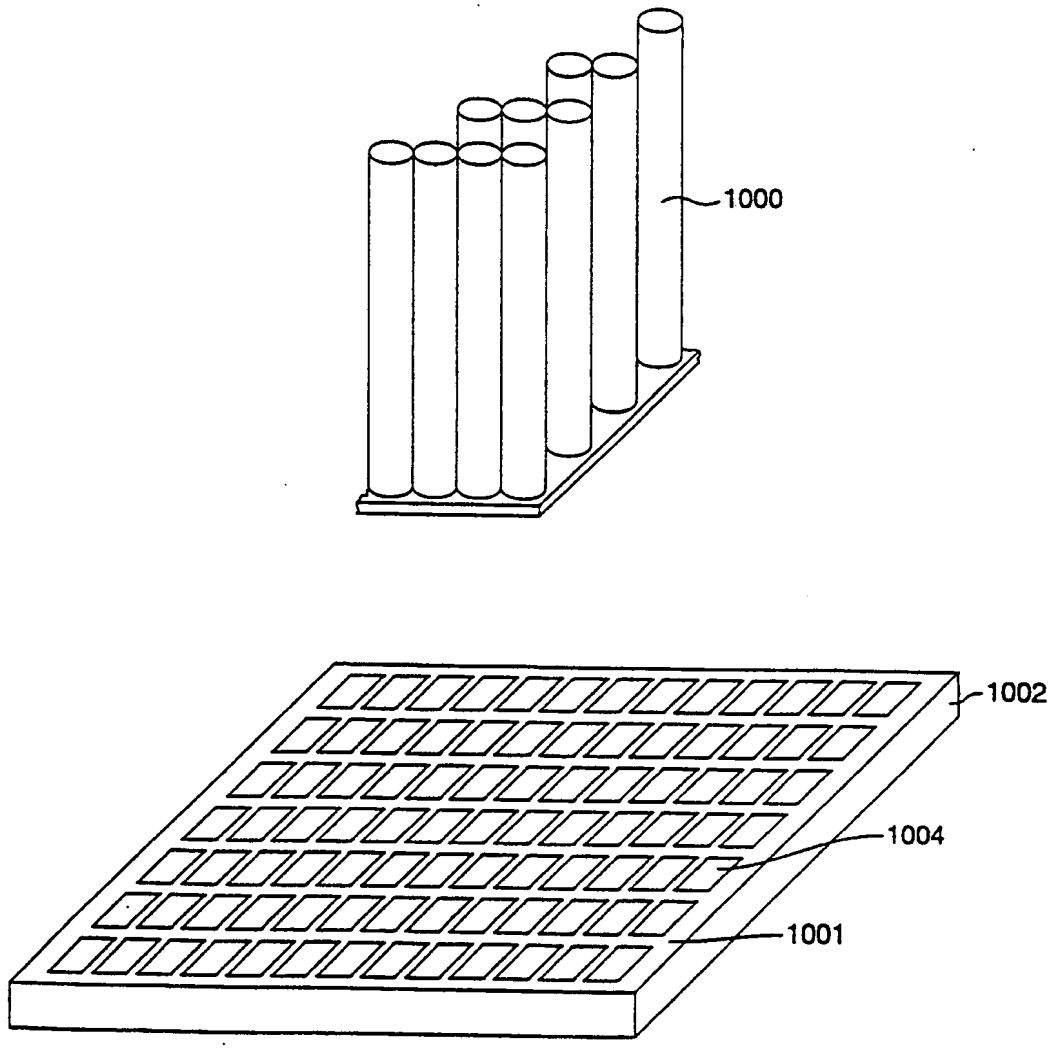



FIG. 12

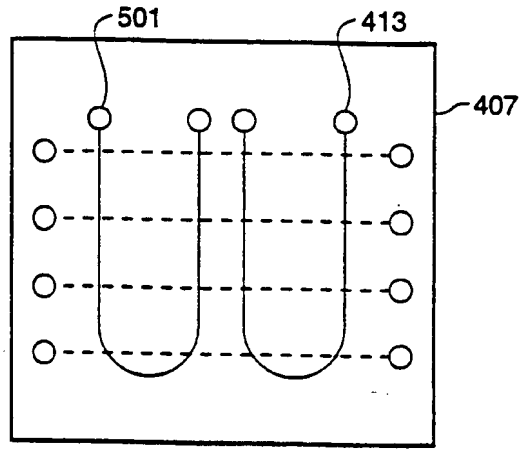



FIG. 13A

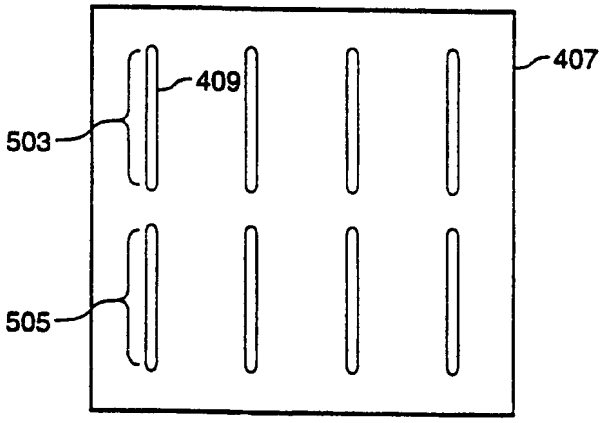
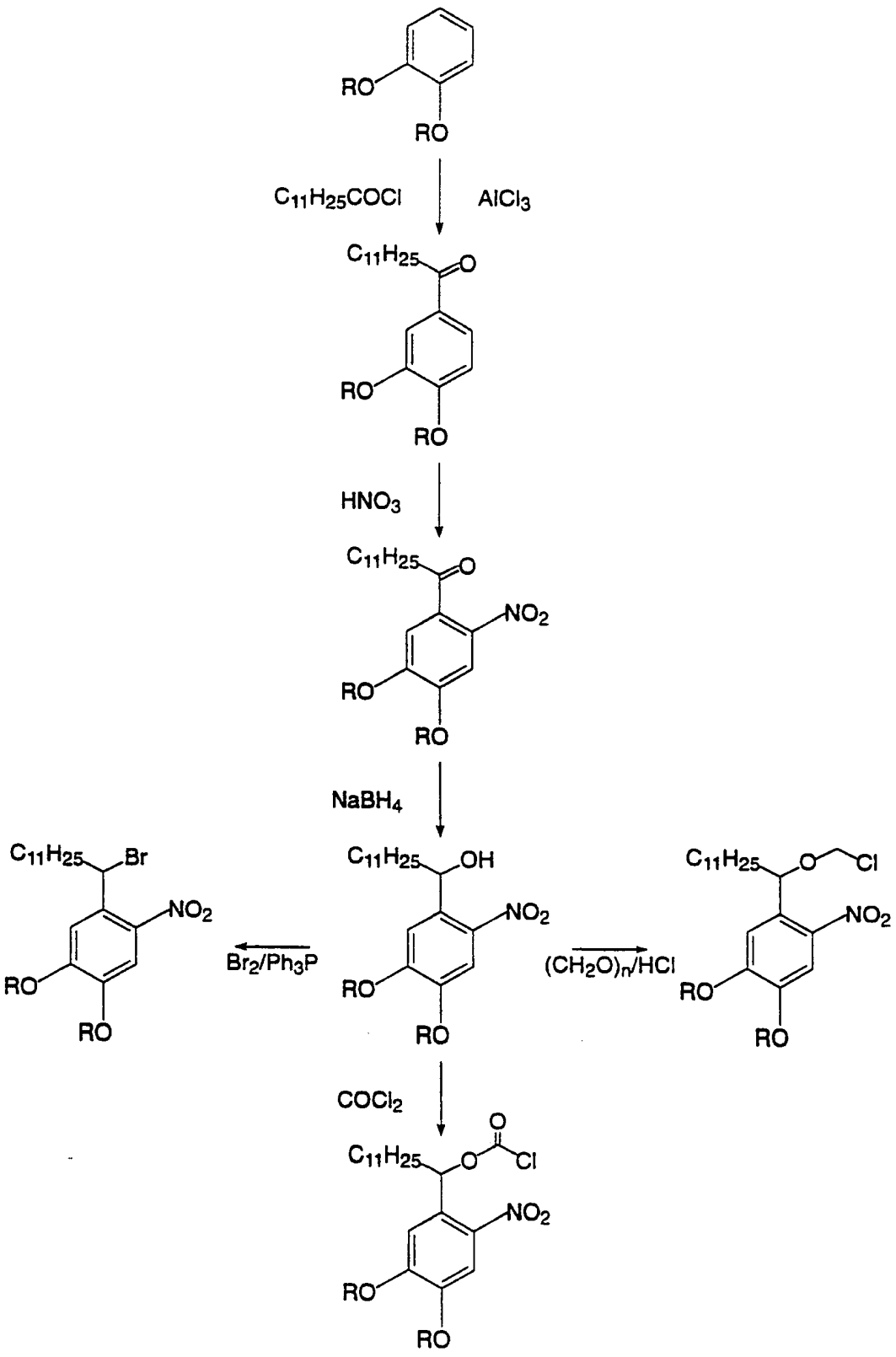



FIG. 13B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RO
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$C_{11}H_{25}$
O
RO
RO
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$NO_2$
$NaBH_4$
$C_{11}H_{25}$
Br
OH
Cl
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$(CH_2O)_n/HCl$
$COCl_2$

*FIG. 14*

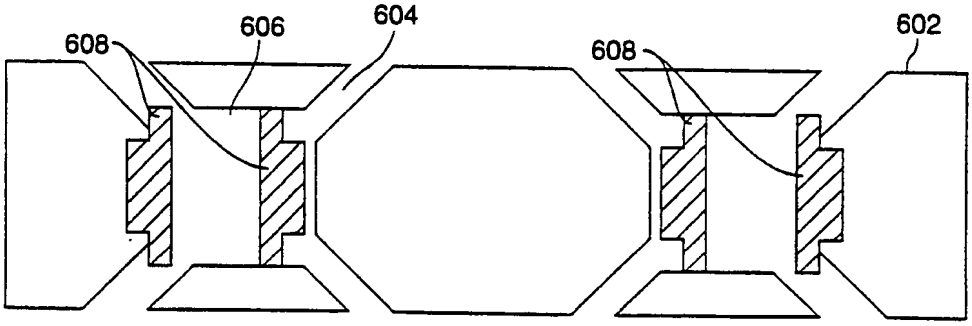


FIG. 15A

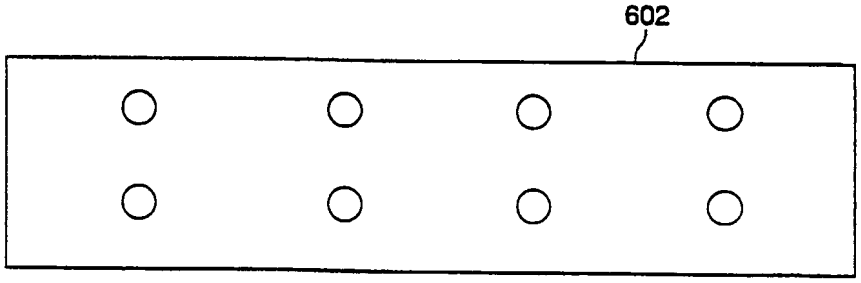


FIG. 15B

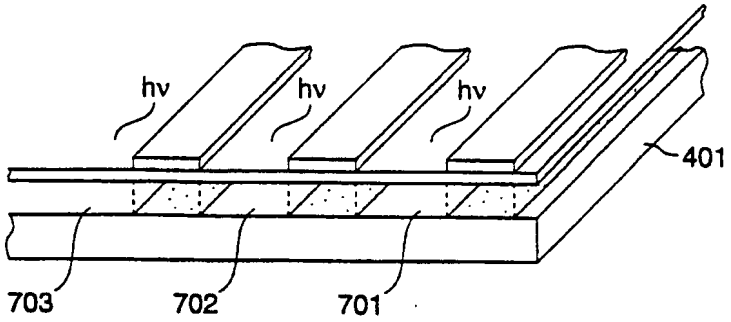


FIG. 16A

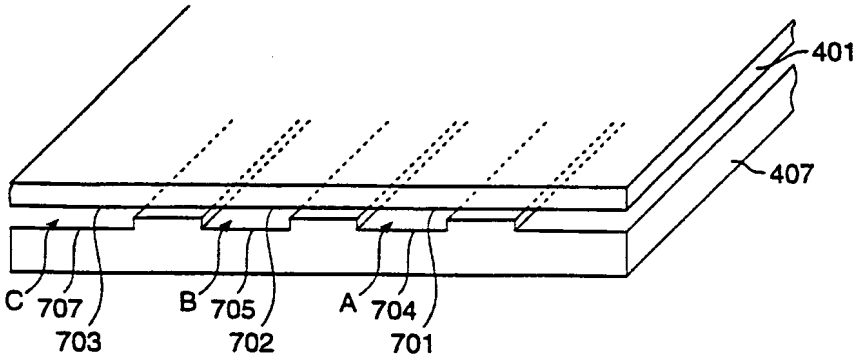


FIG. 16B

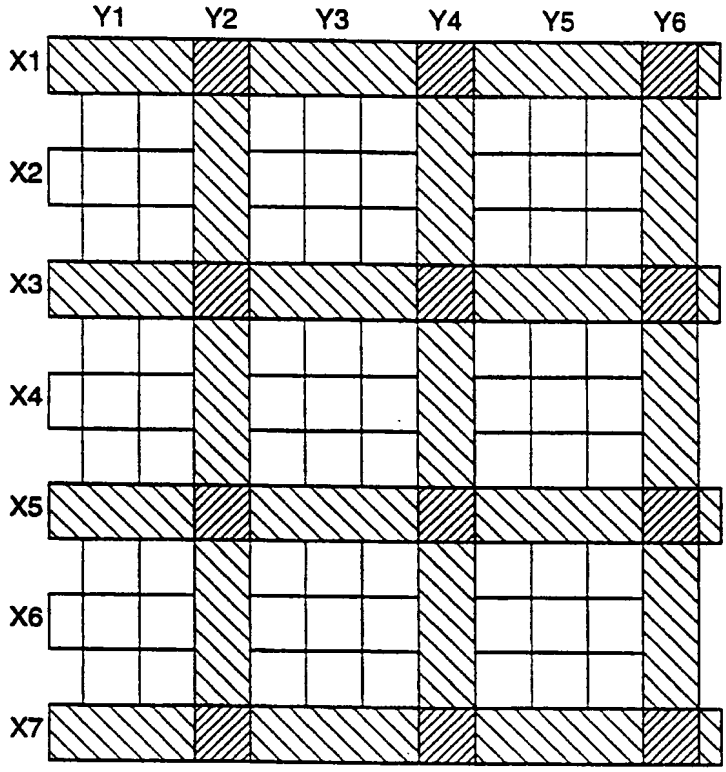


FIG. 17

# INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US92/10183

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG
search terms: peptide, oligonucleotide, solid phase synthesis, channel, light, photosensitive, photolabile, photoremovable

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | SCIENCE, volume 251, issued 15 February 1991, S. Fodor et al., "Light-Directed, Spatially Addressable Parallel Chemical Synthesis", pages 767-773, see entire document. | 1, 3-9, 15-16, 26-27, 29-37<br>2, 10-14, 17-25, 28, 38-47 |
| X,&<br>Y | WO, A, 90/15070 (PIRRUNG) 13 December 1990, see page 6, lines 1-35; page 13, line 21 - page 14, line 16; page 16, line 14 - page 17, line 31; page 19, line 9-12; page 21, line 35 - page 22, line 5; page 24, line 20 - page 25, line 8; page 27, lines 25-37; page 28, lines 21-33; page 29, line 10 - page 31, line 6; page 35, line 23 - page 36, line 16; Figs. 8a/8b; claims 1-46. | 1-10, 14-23, 26-38, 40-47<br>11-13, 24-25, 39 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>14 JANUARY 1993 | Date of mailing of the international search report<br>02 FEB 1993 |
| Name and mailing address of the ISA/<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. NOT APPLICABLE | Authorized officer<br>CAROL A. SPIEGEL<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

# INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US92/10183

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X, & Y | WO, A,91/07087 (AFFYMAX TECHNOLOGIES, N.V.) 30 May 1991, see page 9, line 26 - page 11, line 40; page 12, lines 14-19; page 13, lines 25-27; page 23, lines 17-31; page 30, line 13 - page 31, line 7; page 46, line 25 - page 47, line 28; page 50, line 15 - page 51, line 14; page 51, lines 31-33 | 1, 3-9, 11, 15-16, 26-27, 29-37, 39<br>2, 10, 12-14, 17-25, 28, 38, 40-47 |
| X | US, A, 4,728,502 (Hamill) 01 March 1988, see column 2, line 45 - column 4, line 60. | 18, 21, 24, 25 |
| Y | S.M. SZE, "Lithography", in VLSI TECHNOLOGY, published 1983 by McGraw-Hill Book Company (New York), pages 267-301, see pages 267-274. | 1-47 |
| Y,P | SCIENCE, volume 257, issued 04 September 1992, N. Abbott et al., "Manipulation of the Wettability of Surfaces on the 0.1- to 1-Micrometer Scale Through Micromachining and Molecular Self-Assembly", pages 1380-1382, see entire document. | 1-47 |
| Y,P | JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, volume 114, issued 20 May 1992, S. Bhatia et al., "New Approaches To Producing Patterned Biomolecular Assemblies", pages 4432-4433, see entire document. | 1-47 |
| Y | PROCEEDINGS OF THE INDIAN NATIONAL SCIENCE ACADEMY, volume 53, no. 6, issued 1987, V. Haridasan et al., "Peptide Synthesis Using Photolytically Cleavable 2-Nitrobenzyloxycarbonyl Protecting Group", pages 717-728, see the Introduction, pages 717-718. | 3, 15-16, 19-20, 22-23, 31-32, 40-47 |
| A,E | US, A, 5,175,209 (Beattie et al) 29 December 1992, see entire document | 1-47 |

# INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US92/10183

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

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(54) Title: SPATIALLY-ADDRESSABLE IMMOBILIZATION OF ANTI-LIGANDS ON SURFACES

(57) Abstract

Methods and compositions are described for immobilizing anti-ligands, such as antibodies or antigens, hormones or hormone receptors, oligonucleotides, and polysaccharides on surfaces of solid substrates for various uses. The methods provide surfaces covered with caged binding members which comprise protecting groups capable of being removed upon application of a suitable energy source. Spatially addressed irradiation of predefined regions on the surface permits immobilization of anti-ligands at the activated regions on the surface. Cycles of irradiation on different regions of the surface and immobilization of different anti-ligands allows formation of an immobilized matrix of anti-ligands at defined sites on the surface. The immobilized matrix of anti-ligands permits simultaneous screenings of a liquid sample for ligands having high affinities for certain anti-ligands of the matrix. A preferred embodiment of the invention involves attaching photoactivatable biotin derivatives to a surface. Photolytic activation of the biotin derivatives forms biotin analogs having strong binding affinity for avidin. Biotinylated anti-ligands can be immobilized on activated regions of the surface previously treated with avidin.

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# SPATIALLY-ADDRESSABLE IMMOBILIZATION OF ANTI-LIGANDS ON SURFACES

## BACKGROUND OF THE INVENTION

The present invention relates generally to methods and compositions useful for immobilizing anti-ligands on surfaces. The immobilized anti-ligands, which can be, for example, hormones or hormone receptors, antibodies or antigens, oligosaccharides, and oligonucleotides, may be used in a variety of screening and assay methodologies for ligands in liquid media.

Certain biological molecules are known to interact and bind to other molecules in a very specific manner. Essentially any molecules having a high binding specificity for each other can be considered a ligand/anti-ligand pair, e.g., a vitamin binding to a protein, a cell-surface receptor binding to a hormone or drug, a glycoprotein serving to identify a particular cell to its neighbors, an IgG-class antibody binding to an antigenic determinant, an oligonucleotide sequence binding to its complementary fragment of RNA or DNA, and the like.

The specific binding properties of anti-ligands for ligands have implications for many fields. For example, the strong binding affinity of antibodies for specific determinants on antigens is critical to the field of immunodiagnostics. Additionally, pharmaceutical drug discovery, in many cases, involves discovering novel drugs having desirable patterns of specificity for naturally-occurring receptors or other biologically important anti-ligands. Many other areas of research exist in which the selective interaction of anti-ligands for ligands is important and are readily apparent to those skilled in the art.

The immobilization of anti-ligands onto surfaces is an important step in performing repetitive assays and

screenings of ligands with solid phase systems. Previous methods of attaching anti-ligands to surfaces are limited by low reaction efficiencies or by a general inability to regionally and selectively attach a plurality of anti-ligands to the surface.

A large variety of methods are known for attaching biological molecules to solid supports. See generally, Affinity Techniques. Enzyme Purification: Part B. Methods in Enzymology, Vol. 34, ed. W.B. Jakoby, M. Wilchek, Acad. Press, NY (1974) and Immobilized Biochemicals and Affinity Chromatography, Advances in Experimental Medicine and Biology, vol. 42, ed. R. Dunlap, Plenum Press, NY (1974), which are incorporated herein by reference. For example, U.S. Patent No. 4,681,870 describes a method for introducing free amino or carboxyl groups onto a silica matrix. These groups may subsequently be covalently linked to, e.g., a protein or other anti-ligand, in the presence of a carbodiimide. Alternatively, a silica matrix may be activated by treatment with a cyanogen halide under alkaline conditions. The anti-ligand is covalently attached to the surface upon addition to the activated surface. Another example is presented in U.S. Patent No. 4,282,287, which describes a method for modifying a polymer surface through the successive application of multiple layers of biotin, avidin and extenders. Also, U.S. Patent No. 4,762,881 describes a method for attaching a polypeptide chain to a solid substrate by incorporating a light-sensitive unnatural amino acid group into the polypeptide chain and exposing the product to low-energy ultraviolet light.

Similarly, a variety of techniques have been developed for attaching oligonucleotides to surfaces. For example, U.S. Patent No. 4,542,102 describes a method employing a photochemically active reagent (e.g., a psoralen compound) and a coupling agent, which attaches the photoreagent to the substrate. Photoactivation of the photoreagent binds a nucleic acid sequence to the substrate to give a surface-bound probe for a complementary oligonucleotide of the sequence. However, this method has low quantum yields in protic solvents, lacks

spatial directability, and relies upon initial affinity between the photoreagent and nucleic acids prior to photoactivation.

U.S. Patent No. 4,562,157 describes a technique for attaching biochemical ligands to surfaces by attachment of a photochemically reactive arylazide. Irradiation of the azide creates a reactive nitrene which reacts irreversibly with macromolecules in solution resulting in the formation of a covalent bond. The high reactivity of the nitrene intermediate, however, results in both low coupling efficiencies and many potentially unwanted products due to nonspecific reactions.

Thus, there exists a need for improved methods for attaching a broad range of anti-ligands to predefined regions of a solid support surface. The methods should efficiently provide stable attachment of selected anti-ligands to the activated surface regions, yet attachment should be restricted to the activated regions. The present invention fulfills these and other needs.

## SUMMARY OF THE INVENTION

Novel methods and compositions of matter are provided for immobilizing anti-ligands on predefined regions of a surface of a solid support. The methods involve attaching to the surface a caged binding member which has a relatively low affinity for other potentially binding species, such as anti-ligands and specific binding substances. The caged binding member is convertible, e.g., by irradiation, to a binding member ultimately capable of immobilizing a desired anti-ligand, preferably via a non-covalent interaction. Predefined regions of the surface are selectively irradiated to convert the caged binding members in the predefined regions to activated binding members. The desired anti-ligands subsequently can be immobilized on the activated regions of the surface.

Importantly, the spatial addressability afforded by the method of the present invention allows the formation of patterned surfaces having preselected reactivities. For example, by using lithographic techniques known in the

semiconductor industry, light can be directed to relatively small and precisely known locations on the surface. It is, therefore, possible to activate discrete, predetermined locations on the surface for attachment of anti-ligands. The resulting surface will have a variety of uses. For example, direct binding assays can be performed in which ligands can be simultaneously tested for affinity at different anti-ligands attached to the surface. Ligand binding is detected by a technique such as autoradiography when the ligand is radioactively labelled. Alternatively, fluorescence or other optical techniques can be used. By determining the locations and intensities of labels on the surface it is possible to simultaneously screen ligands for affinity to a plurality of anti-ligands.

A further understanding of the nature and advantages of the invention may be realized by reference to the remaining portions of the specification.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 presents chromatographic results showing that NVOC-biotin-ONP is converted to biotin-ONP upon illumination in solution.

FIG. 2 presents radioligand binding results showing NVOC-biotin-OMe has low affinity for avidin prior to illumination but high affinity after illumination in solution.

FIG. 3 presents gamma counting results showing that illumination of membrane-bound NVOC-biotin increases the binding of radioactive avidin to the membrane.

FIG. 4 presents fluorescence results showing the spatial immobilization of Fluorescein-Streptavidin on a biotinylated surface.

FIG. 5 presents fluorescence results showing the spatial immobilization of Fluorescein-Biotin on a surface modified with Streptavidin.

FIG. 6 presents fluorescence results showing the spatial immobilization of Fluorescein-Streptavidin on a surface having biotin bound by a polyether linker.

FIG. 7 presents fluorescence results showing the spatial immobilization of Fluorescein-Streptavidin on a biotinylated surface.

FIG. 8 presents fluorescence results showing the spatial immobilization of Bodipy-Streptavidin on a biotinylated surface.

FIG. 9 presents fluorescence results showing the effect of inserting linkers of different lengths on the binding of Fluorescein-Streptavidin.

FIG. 10a presents fluorescence results showing binding of Fluorescein-anti-Rabbit IgG to a slide having multiple anti-ligands.

FIG. 10b presents fluorescence results showing binding of Fluorescein-anti-Mouse IgG to a slide having multiple anti-ligands.

FIG. 10c presents fluorescence results showing binding of a mixture of Fluorescein-anti-Rabbit IgG and Fluorescein-anti-Mouse IgG to a slide having multiple anti-ligands.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

CONTENTS:



### I. Glossary

The following terms have the following meanings and abbreviations as used herein:

1. Surface (S): A surface is any generally two-dimensional structure on a solid substrate. A surface may have steps, ridges, kinks, terraces and the like without ceasing to be a surface.

2. Predefined Region ($S_i$): A predefined region is a localized area on a surface which is or is intended to be

activated. The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, etc.

3. Crosslinking Group (X): A crosslinking group is a bifunctional chemical entity that serves to connect a binding member to a surface. Usually, crosslinking groups will be heterobifunctional, i.e., they will have different chemical reactivities on either end of the linking group.

4. Binding Member (B): A binding member is any substance having a sufficiently high affinity for another substance. A binding member will have a sufficiently high affinity for another substance for practice of this invention when it effectively binds the substance without irreversibly separating from it throughout the handling and performance steps of the invention. A binding member is usually, but not always, connected to a surface via a crosslinking group.

5. Caged Binding Member (B*): A caged binding member is a binding member that is provided with a removable (labilizable) chemical protecting group. Such protecting groups are characterized by their abilities to deter effectively binding between the binding member to which they are attached and other substances otherwise having affinity for the binding member. Also, the protecting groups are readily labilizable, i.e., they can be detached from the binding member to which they are attached upon exposure to a suitable source of energy.

6. Specific Binding Substance (SBS): A specific binding substance is a compound having a sufficiently high affinity and selectivity for binding to a binding member to permit practice of the present invention. A specific binding substance may be larger or smaller than the binding member to which it specifically binds. The specific binding substance serves as a bridge for attaching an anti-ligand to binding members on the surface.

7. Anti-ligand ($AL_i$): An anti-ligand is a molecule that has a known or unknown affinity for a given ligand and can be immobilized on a predefined region of the surface. Anti-ligands may be naturally-occurring or manmade molecules. Also, they can be employed in their unaltered state or as

aggregates with other species. Anti-ligands may be reversibly attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. By "reversibly attached" is meant that the binding of the anti-ligand (or specific binding member or ligand) is reversible and has, therefore, a substantially non-zero reverse, or unbinding, rate. Such reversible attachments can arise from noncovalent interactions, such as electrostatic forces, van der Waals forces, hydrophobic (i.e., entropic) forces, and the like. Furthermore, reversible attachments also may arise from certain, but not all covalent bonding reactions. Examples include, but are not limited to, attachment by the formation of hemiacetals, hemiketals, imines, acetals, ketals, and the like (See, Morrison et al., "Organic Chemistry", 2nd ed., ch. 19 (1966), which is incorporated herein by reference). Examples of anti-ligands which can be employed by this invention include, but are not restricted to, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells or other materials), hormones, drugs, oligonucleotides, peptides, enzymes, substrates, cofactors, lectins, sugars, oligosaccharides, cells, cellular membranes, and organelles.

8. Ligand (L): A ligand is a solvated molecule that is recognized by a particular anti-ligand. Examples of ligands that can be investigated by this invention include, but are not restricted to agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, oligosaccharides, proteins, and monoclonal antibodies.

## II. Overview

The present invention provides methods for forming predefined regions on a surface of a solid support, wherein the predefined regions are capable of immobilizing anti-ligands. The methods make use of caged binding members attached to the surface to enable selective activation of the predefined

regions. The caged binding members are converted to binding members ultimately capable of binding anti-ligands upon selective activation of the predefined regions. The activated binding members are then used to immobilize anti-ligands on the predefined region of the surface. The above procedure can be repeated at the same or different sites on the surface so as to provide a surface prepared with a plurality of regions on the surface containing the same or different anti-ligands. When the anti-ligands have a particular affinity for one or more ligands, screenings and assays for the ligands can be conducted in the regions of the surface containing the anti-ligands.

The present methods are distinguished by the employment of novel caged binding members attached to the substrate. Caged (unactivated) members have a relatively low binding affinity for anti-ligands or specific binding substances when compared with the corresponding affinities of activated binding members. Thus, the binding members are protected until a suitable source of energy is applied to the regions of the surface desired to be activated. Upon application of a suitable energy source, the caging groups labilize, thereby presenting the activated binding member. A typical energy source will be light.

Once the binding members on the surface are activated they may be attached to an anti-ligand. The anti-ligand chosen may be a monoclonal antibody, a nucleic acid sequence, a drug receptor, etc. The anti-ligand will usually, though not always, be prepared so as to permit attaching it, directly or indirectly, to a binding member. For example, a specific binding substance having a strong binding affinity for the binding member and a strong binding affinity for the anti-ligand may be used as a bridge. Alternatively, a covalently-linked conjugate of the specific binding substance and anti-ligand may be used. The method uses an anti-ligand prepared such that the anti-ligand retains its activity toward a particular ligand.

Preferably, the caged binding member attached to the solid substrate will be a photoactivatable biotin analog, i.e., a biotin molecule that has been chemically modified with

photoactivatable protecting groups so that it has a significantly reduced binding affinity for avidin or avidin analogs compared to that of natural biotin. In a preferred embodiment, the protecting groups localized in a predefined region of the surface will be removed upon application of a suitable source of radiation to give binding members, that are biotin or a functionally analogous compound having substantially the same binding affinity for avidin or avidin analogs as does biotin.

In another preferred embodiment, avidin or an avidin analog will be incubated with activated binding members on the surface until the avidin binds strongly to the binding members. The avidin so immobilized on predefined regions of the surface, can then be incubated with a desired anti-ligand or conjugate of a desired anti-ligand. The multiple biotin binding sites on avidin allow simultaneous binding of biotin attached to the surface and biotin attached to the anti-ligand. The anti-ligand will preferably be biotinylated, e.g., a biotinylated antibody, when avidin is first immobilized on the predefined regions of the surface. Alternatively, a preferred embodiment will present an avidin/biotinylated anti-ligand complex, which has been previously prepared, to activated binding members on the surface.

The following equations depict the best modes of practicing the invention:

The attachment of binding members (B) to a surface (S) of a solid substrate is illustrated by the following reactions:

S + B* --> S-B* or

S + B --> S-B (1)
S-B + * --> S-B* (2)

where "*" represents a protecting (caging) group. B* is a caged binding member. The protecting groups can either be attached to the binding members once they have been attached to the surface, or more preferably, they will be attached to binding members prior to attaching the binding members to the surface.

Also, surface attachment of binding members can be effected through the use of crosslinking groups (X). This is represented by the following reactions:

$$S + X \rightarrow S\text{-}X \quad (1)$$
$$S\text{-}X + B^* \rightarrow S\text{-}X\text{-}B^* \quad (2) \text{ or}$$

$$S\text{-}X + B \rightarrow S\text{-}X\text{-}B \quad (2')$$
$$S\text{-}X\text{-}B + * \rightarrow S\text{-}X\text{-}B^*$$

The crosslinking groups will usually, though not always, be heterobifunctional chemical species having a first reactivity which permits the crosslinking group to bind readily to the surface and a second reactivity which permits the crosslinking group to bind readily with binding members.

Predefined regions ($S_i$) on the surface can be activated for ultimate immobilization of anti-ligands in the predefined regions by selectively irradiating predefined regions to convert photoactivatable binding members in the predefined region to binding members. This process is illustrated by the following reactions:

$$Si\text{-}B^* \xrightarrow{hv} Si\text{-}B + * \text{ or}$$

$$Si\text{-}X\text{-}B^* \xrightarrow{hv} Si\text{-}X\text{-}B + *.$$

The free protecting group, "*", may or may not undergo decomposition reactions. It will usually be washed from the surface, depending upon whether it interferes with subsequent reactions.

Immobilization of anti-ligands ($AL_i$) on predefined regions of the surface can be effected by binding the anti-ligands directly to binding members or through a bridging specific binding substance (SBS). The specific binding substance may be introduced to binding members alone or as a previously prepared conjugate of the anti-ligand. Multiple anti-ligands may be immobilized on the surface when the specific binding substance contains multiple binding sites. Also, it should be noted that an advantage of using a specific binding substance is that an immobilization technique generic for many anti-ligands may be employed. Immobilization of

anti-ligands on predefined regions of the surface is illustrated by the following reactions:

$$S_i\text{-X-B} + AL_i \text{ --> } S_i\text{-X-B-}AL_i \text{ or}$$

$$S_i\text{-X-B} + \text{SBS-}AL_i \text{ --> } S_i\text{-X-B-SBS-}AL_i \text{ or}$$

$$S_i\text{-X-B} + \text{SBS} \text{ --> } S_i\text{-X-B-SBS} \quad (1)$$

$$S_i\text{-X-B-SBS} + \text{ALi} \text{ --> } S_i\text{-X-B-SBS-}AL_i \quad (2),$$

where the horizontal lines B-SBS, B-$AL_i$, or SBS-$AL_i$ represent bonding between two molecules, preferably a non-covalent bond.

An example of immobilizing a different anti-ligand ($AL_j$) on a different predefined region ($S_j$) of the surface is shown by the equation:

$$S_j\text{-X-B} + \text{SBS-}AL_j \text{ --> } S_j\text{-X-B-SBS-}AL_j.$$

Repetition of the above steps on different regions of the surface can produce a matrix of anti-ligands immobilized on the surface. Such a matrix can have any desired pattern of anti-ligands. An example of such a matrix is given below:

| | | |
|---|---|---|
| $AL_i$ | $AL_j$ | B* |
| B* | B* | $AL_k$ |
| $AL_l$ | B* | B*. |

An immobilized anti-ligand on a surface will have a specific binding affinity for a particular ligand (L). An example of a direct assay on a predefined region of the surface for the presence of a labeled ligand (L') in a liquid medium is illustrated by the following reaction:

$$S_i\text{-X-B-SBS-}AL_i + L' \text{ --> } S_i\text{-X-B-SBS-}AL_i\text{-}L'.$$

The resulting surface can be washed free of unbound ligand and analyzed for the presence of label. The labels will provide markers localized at the predefined regions on the surface corresponding to the presence of anti-ligands for the ligand at those predefined regions.

Some examples of competitive assays, in which a target ligand (L) "competes" with another ligand (L') for a site on the surface, are illustrated by the following reactions:

$$S_i\text{-X-B-SBS-AL}_i\text{-L'} + L \rightarrow S_i\text{-X-B-SBS-AL}_i\text{-L} + L' \quad (1).$$
$$S_i\text{-X-B-SBS-AL}_i + L/L' \rightarrow S_i\text{-X-B-SBS-AL}_i\text{-L'} + L \quad (2).$$

The presence of target ligand can be determined by analyzing appropriately for the loss or buildup of label on the predefined regions of the surface.

III. Substrate Preparation

Essentially, any conceivable solid substrate may be employed in the invention. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate and its surface should also be chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, $SiO_2$, $SiN_4$, modified silicon, or any one of a wide variety of polymers such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, or combinations thereof. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or single-crystal silicon with surface features of less than 10 Å.

Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, etc., provided only that

caged binding members can be attached firmly to the surface of the substrate. Preferably, the surface will contain reactive groups, which could be carboxyl, amino, hydroxyl, or the like. Most preferably, the surface will be optically transparent and will have surface Si-OH functionalities, such as are found on silica surfaces.

The surface of the substrate is preferably provided with a layer of crosslinking groups, although it will be understood that the crosslinking groups are not required elements of the invention. The crosslinking groups are preferably of sufficient length to permit binding members on the surface to interact freely with compounds in solution. Crosslinking groups may be selected from any suitable class of compounds, for example, aryl acetylenes, ethylene glycol oligomers containing 2-10 monomer units, diamines, diacids, amino acids, or combinations thereof. Other crosslinking groups may be used in light of this disclosure.

Crosslinking groups may be attached to the surface by a variety of methods which are readily apparent to one having skill in the art. For example, crosslinking groups may be attached to the surface by siloxane bonds formed via reactions of crosslinking groups bearing trichlorosilyl or trisalkoxy groups with hydroxyl groups on the surface of the substrate. Preferably, the crosslinking group used with a glass surface is N-BOC-aminopropyltriethoxy silane. The crosslinking groups may optionally be attached in an ordered array, i.e., as parts of the head groups in a polymerized Langmuir Blodgett film. Clearly, the type of crosslinking group selected, and the method selected for attaching it to the surface, will depend primarily on the crosslinking group having suitable reactivity with the binding member desired to be attached to the surface.

Additional length may be added to the crosslinking groups by the addition of single or multiple linking groups. Such linking groups are preferably heterobifunctional, having one end adapted to react with the crosslinking groups and the other end adapted to react with the binding member or an another linking group. The linking groups may be attached by a variety of methods which are readily apparent to one skilled in

the art. For instance, esterification or amidation reactions of an activated ester of the linking group with a reactive hydroxyl or amine on the free end of the crosslinking group. A preferred linking group is N-BOC-6-aminocaproic acid (i.e., N-BOC-6-aminohexanoic acid) attached by the BOP-activated ester. After deprotection to liberate the free amine terminus, another N-BOC-aminocaproic linker can be added. Attachment of crosslinking and linking groups to caged binding members are discussed more fully below.

Many methods are available for immobilizing the binding members of the present invention on surfaces. The binding members may be linked to the surface in their active forms, and later provided with protecting (caging) groups. More preferably, binding members will be provided in their protected forms. The method chosen for linking binding members to the surface will depend upon the chemical properties of the binding member selected for attachment to the surface. A preferred method for immobilizing the binding members of the present invention involves chemical derivatization or activation of the caged binding member prior to attachment to the surface or linker. This derivative or activated species is then reacted with functionalities on the substrate to give the desired linkage. For example, one method for attaching a binding member to a surface employs a heterobifunctional crosslinking reagent, such as diepoxide, which both activates the surface and provides a group that reacts with an activated binding member. Alternatively, the surface can be activated with cyanogen bromide. Reaction with a binding member containing a terminal amino group permits attachment of the binding member to the surface. (U.S. Patent No. 4,542,102). In the presence of a carbodiimide or other activating agent, for example, the amine group can be coupled to the carboxyl terminus of a binding member desired to be immobilized on the surface.

A preferred embodiment of the present invention involves attaching "caged" derivatives of biotin or biotin analogs to a glass surface. Caged biotin may be attached to the surface through strong noncovalent interactions, e.g., by

crosslinking via a suitable linker to another biotin molecule and reacting with a surface to which avidin has been attached, or alternatively, and preferably, by covalent attachment to the surface. The latter may be accomplished by derivatizing caged-biotin and biotin analogues at their carboxylic acid terminus. Many biotin derivatives have been described previously. For example, the surface can be provided with biotin anti-ligands, e.g., antibiotin antibodies, which specifically bind the carboxyl arm of biotin without interfering with the avidin-binding ureido ring of biotin.

Still another method for immobilizing the caged binding members of the present invention involves chemical derivatization or activation of the binding member prior to attachment to the surface or linker. For example, when the surface is a polymer containing primary amines and biotin is selected as the binding member, the N-hydroxysuccinimide ester derivative of biotin can react with the surface to give a biotin-surface complex (U.S. Patent No. 4,282,287).

Alternatively, and preferably, photoactivatable biotin and biotin analog derivatives will be covalently attached to the surface. To effect this transformation, the biotin and biotin analogs may be derivatized at their carboxylic acid terminus. Many biotin derivatives have been described previously involving derivatization at the free carboxyl end of biotin. See, e.g., Bayer et al., Methods of Biochemical Analysis, vol. 26 (D. Glick, ed.), 1-45 (1980), which is incorporated herein by reference. For example, photoactivatable biotin derivatives may be reacted, in the presence of an activating reagent, such as a carbodiimine or BOP, with the amine groups of crosslinking groups previously immobilized on the surface to give the biotin attached to the surface via an amide linkage. The active ureido ring of biotin, either free or protected, is located far enough away from the site of attachment that, when unprotected, binding with avidin is not significantly diminished.

It should be appreciated that the above discussion of exemplary surface attachment reactions is only illustrative of the general method for attaching caged binding members to a

surface and should not be regarded in any way as limiting the applicability of the method to biotin, biotin analogs, or specific crosslinking groups. Other types of binding members also amenable to the above attachment techniques include enzymes, antibodies, oligonucleotides and the like and are readily apparent to one skilled in the art.

IV. Properties of Binding Members and Caging Groups

The present method permits use of a wide variety of caged binding members to effect the immobilization of anti-ligands on the surface. The method is generally applicable to such classes of compounds as enzymes, substrates, cofactors, immunoglobulins, antibodies, haptens, antigens, oligonucleotides, oligosaccharides, lectins, proteins, glycoproteins, etc., being the binding member provided that the selected derivative of such species is activatable upon exposure to a suitable energy source. Moreover, the binding member can possess a multiplicity of binding sites for an anti-ligand or specific binding substance.

The binding member selected will have a high binding affinity either for an anti-ligand or a specific binding substance. Preferably, a specific binding substance will provide a link between the binding member and the anti-ligand. Usually, the interactions between a binding member and a specific binding substance and an anti-ligand or anti-ligand conjugate will be noncovalent in nature. When a specific binding substance provides a link between the binding member and the anti-ligand, the specific binding substance will be connected to the anti-ligand either covalently or through noncovalent interactions.

The binding member on a surface must have a strong affinity for an anti-ligand or specific binding substance to prevent migration or loss of the anti-ligand during wash steps. The affinity between the binding member and an anti-ligand or a specific binding substance is best represented by the off-rate of anti-ligand or specific binding substance from a binding member. However, off-rates often are not conveniently known or determined. Therefore, binding affinity may also be

represented by the affinity constant ($K_a$) for equilibrium concentrations of associated and dissociated configurations; i.e., $K_a$ = [B-SBS]/[B][SBS], where [B], [SBS] and [B-SBS] are the concentrations of the binding member (B), the concentration of specific binding substance (SBS), and the concentration of associated complex (B-SBS), respectively, at equilibrium. An analogous definition of $K_a$ applies when SBS is replaced with an anti-ligand (AL) or a specific binding substance - anti-ligand conjugate (SBS-AL), etc. The affinity constants of some sample classes of compounds suitable for use in the present invention are presented in Table 1.

Table 1

Affinities of Sample Binding Members and Specific Binding Substances (SBS).

| Binding Member | SBS | Affinity($K_a$ $M^{-1}$) |
|---|---|---|
| Membrane sites | Lectins | $10^{6-7}$ |
| Haptens | Antibodies | $10^{5-11}$ |
| Antigenic determinants | Antibodies | $10^{5-11}$ |
| Biotin | Avidin | $10^{15}$ |
| Iminobiotin | Avidin | $10^{11}$ |
| 2-thiobiotin | Avidin | $10^{13}$ |
| Dethiobiotin | Avidin | $10^{13}$ |
| 1'-N-methoxy-carbonylbiotin methyl ester | Avidin | $10^{7}$ |
| 3'-N-methoxy-carbonylbiotin methyl ester | Avidin | $10^{9}$ |

*References: U.S. Patent No. 4,282,287; Green, "Avidin" in Advances in Protein Chemistry, Academic Press, vol.29, 105 (1975).

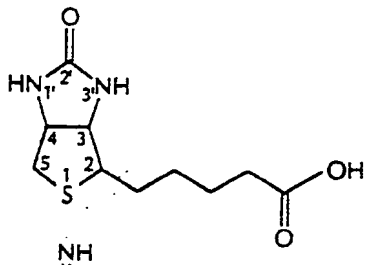
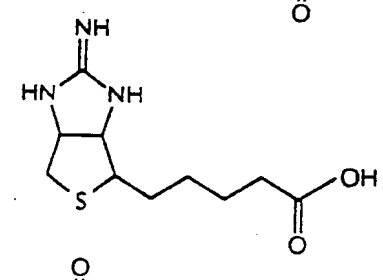
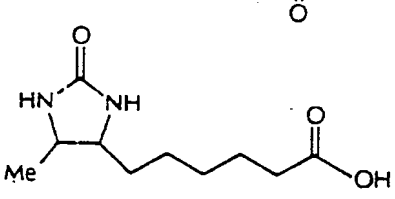
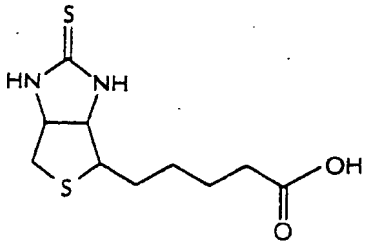
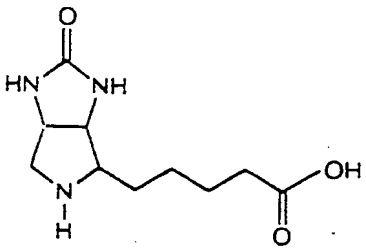
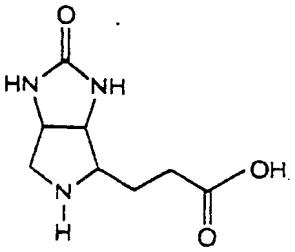
Preferably, the affinity constant between the activated binding member and another species, i.e., a specific binding species, an anti-ligand, or anti-ligand conjugate, will be greater than about $10^7$ $M^{-1}$. More preferably, the $K_a$ will be greater than about $10^{11}$ $M^{-1}$, and most preferably, the $K_a$ will be about $10^{15}$ $M^{-1}$ or greater. Likewise, when a specific binding substance is used, the affinity constant between the specific binding substance and an anti-ligand or anti-ligand conjugate will have substantially the same ranges as given above.

An activated (uncaged) binding member is considered to have a relatively strong (high) binding affinity for another species, i.e., a specific binding substance, an anti-ligand, or a conjugate of an anti-ligand, when the $K_a$ between the binding member and the other species is at least about three orders of magnitude greater than the corresponding $K_a$ between the caged binding member and the other species. Similarly, a caged binding member is considered to have a relatively low binding affinity for another species, i.e., specific binding substance, anti-ligand or anti-ligand conjugate, when the $K_a$ between the caged binding member and the other species is about three orders of magnitude less than the corresponding $K_a$ for the activated binding member. Preferably, the affinity constant for the caged binding member will be at least five orders of magnitude lower than the corresponding activated binding member's affinity constant. Most preferably, the binding constant for the caged binding member will be even lower, e.g., seven orders of magnitude lower, than the corresponding activated binding member's affinity constant. However, the suitability of a given caged binding member/ binding member pair for practice of the invention is determined ultimately by whether the selected pair permits proper operation of the invention.

A preferred embodiment of the present invention employs biotin and biotin analogs as the binding members. Typical biotin analogs include dethiobiotin, iminobiotin, 2-thiobiotin, azabiotin, biocytin, and biotin sulfone, and other compounds readily apparent to one skilled in the art. Exemplary biotin analogs include, but are not limited by, those presented in Table 2. Other biotin analogs are presented in N. Green, "Avidin", in Advances in Protein Chemistry, Vol. 29, Acad. Press, p. 85-133 (1975), which is incorporated by reference herein. Biotin analogs include compounds and structures in which biotin is bound to another species, such as a surface, as long as the analog has a binding affinity for avidin that is similar to that of biotin. The biotin or biotin analogs may be subsequently reacted with avidin compounds, streptavidin, and analogues thereof.

Typical examples of avidin and avidin analogs include, but are not limited to, the avidin found in eggs and streptavidin. Streptavidin is a typical example of an avidin analog and is a bacterial biotin-binding protein which has physical characteristics similar to those of egg avidin, despite considerable differences in composition.

Table 2

| Biotin and Biotin Analogs | Name |
|---|---|
|  | Biotin |
|  | Iminobiotin |
|  | Dethiobiotin |
|  | 2'-thiobiotin |
|  | Azabiotin |
|  | Bisnorazabiotin |

Reference: N. Green, "Avidin," in Advances in Protein Chemistry, Vol. 29, Academic Press, p. 85-133 (1975).

Nonbiotin binding members and their corresponding specific binding substances may be employed in the present invention. By way of example and not limitation, some alternative embodiments of the invention follow:

1. Caged Cyclic AMP/ Anti-cAMP Antibodies

High-affinity polyclonal antibodies to cAMP are produced by immunizing with 2'O-monosuccinyl adenosine 3',5' cyclic monophosphate conjugated to a protein such as bovine serum albumin or thyroglobulin. Purified polyclonal antibodies are prepared by affinity chromatography using agarose gel to which 2'O-monosuccinyl adenosine 3',5' cyclic monophosphate has been conjugated. The $K_a$ of polyclonal antibodies to cAMP is in the range of $10^{10}$ to $10^{12}$ $M^{-1}$.

A photoactivatable analog of cAMP has been previously described (Nerbone et al., Nature (1984) 310:74). It is unlikely that polyclonal antibodies against cAMP have high affinity for the photoactivatable analog of cAMP. If the polyclonal antibodies should cross-react with the photoactivatable analog of cAMP, monoclonal antibodies can be produced which discriminate between cAMP and the photoactivatable cAMP analog.

The 2'O-monosuccinyl derivative of the photo-activatable cAMP analog is attached to a surface through the free carboxyl of the succinyl group as described above. Specific regions of the surface are illuminated resulting in the removal of the protecting group from the cAMP. Anti-ligands which have been conjugated to anti-cAMP antibodies are reacted with the surface. The anti-ligands are immobilized only at the predefined regions of the surface that were illuminated.

2. Caged Tetrahydrofolate/ Folate Binding Proteins

$N_5$-(Nitroveratryloxycarbonyl)tetrahydrofolate is activated at its glutamyl gamma-carboxylate with a carbodiimide reagent and coupled to an amino-derivatized surface. Desired predefined regions on the surface are irradiated with light suitable for deprotection of the NVOC group. In the

illuminated regions, the NVOC group is removed to produce tetrahydrofolate bound to the surface. High-affinity folate binding proteins derived from human erythrocyte membranes (Antony et al., J. Clin. Invest. (1987) 80:711-723; $K_a = 3 \times 10^{11}\ M^{-1}$ for tetrahydrofolate), crosslinked to a desired anti-ligand, are then immobilized on the selected regions of the surface.

3. Caged Mannose/ Concanavalin A

8'-(Trichlorosilyl)octyl 6-(nitroveratryloxy)-a-D-mannoside is covalently attached to a silica or glass surface by methods well-known to those skilled in the art. Predefined regions of the surface are irradiated with light suitable for deprotection of the nitroveratryloxy group. In the irradiated regions, the protecting group is removed to produce octyl-α-D-mannoside bound to the surface. In unexposed areas, the 6-(nitroveratryloxy) group protects the mannoside from binding to the lectin. Conconavalin A, crosslinked to a desired anti-ligand, is added to the surface and binds to those mannose units on the surface that have been deprotected. The anti-ligand is thereby immobilized on the desired regions of the surface.

The above modes for practicing the invention are examples only. Those skilled in the art will recognize that any pair of (i) binding member and specific binding substance, (ii) binding member and anti-ligand or anti-ligand conjugate, or (iii) specific binding substance and anti-ligand or anti-ligand conjugate may be used. The only restrictions on the choice of binding member, specific binding substance and anti-ligand or anti-ligand conjugate are that: (1) the binding member has a high affinity for the specific binding substance, anti-ligand or anti-ligand conjugate selected, (2) the binding member can be "caged" with a removable protecting group, and (3) the caged binding member has a relatively low affinity for specific binding substances, anti-ligands, or anti-ligand conjugates, or any other species which interfere with practice of the invention.

A. Caging Groups

Many different protecting (caging) groups can be employed for modifying binding members to give the caged binding members of the present invention. The protecting groups should be sterically large enough to reduce the affinity of the binding member for anti-ligands or specific binding substances to permit operability of the invention, although protecting groups utilizing other types of interactions, such as electronic (i.e., Van der Waals), hydrophobic, etc., could be used. The selection of suitable caging groups will depend upon the size and chemical nature of the binding member chosen and will be readily apparent to one skilled in the art.

In a preferred embodiment, the caging groups will be photoactivatable. The properties and uses of photoreactive caged compounds have been reviewed. See, J. McCray, et al., Annu. Rev. Biophys., Biophys. Chem., 18: 239-70 (1989), which is incorporated herein by reference. Preferably, the photosensitive cages will be activatable by low energy ultraviolet or visible light. In some embodiments, however, activation may be performed by the methods discussed later, including localized heating, electron beam techniques, laser pumping, and oxidation or reduction with microelectrodes. Alternatively, the reactive group may be activatable by electron beam lithography, X-ray lithography, or any other radiation. Suitable reactive groups for electron beam lithography include sulfonyl compounds. Other methods may be used including, for example, exposure to an electric current source, preferably using microelectrodes directed to the predefined regions of the surface which are desired for activation. Other reactive groups and methods of activation may be used in light of this disclosure.

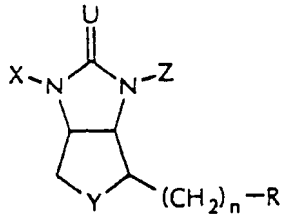
A further preferred embodiment of the present invention employs photoactivatable N-derivatives of biotin and biotin analogs to reduce the natural affinity of biotin for other compounds, such as avidin used as a specific binding substance, until the groups attached to the N-positions are photoremoved. A few references describe N-derivatization of biotin and biotin analogs. See, Kohn et al., J. Org. Chem.

(1977) 42:941-948, and Knappe et al., Biochemische Zeitschrift, 335, 168-176 (1961). However, none of these references provide photoactivatable biotin derivatives. The use of a photosensitive biotin derivative, photobiotin, has been previously described for labelling proteins and nucleic acids. Lacey, E. Anal. Biochem., 163: 151-8 (1987); Forster, A.C. Nucleic Acids Res., 13: 745-61 (1985). However, photobiotin is a derivative of the carboxylate terminus of biotin, which is located away from the recognition site and, hence, does not significantly reduce binding to avidin or streptavidin.

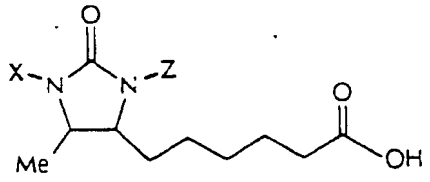
Many, although not all, of the photosensitive protecting groups will be aromatic compounds. Suitable photoremovable protecting groups are described in, for example, McCray, et al., Patchornik, J. Am. Chem. Soc. (1970) 92:6333 and Amit, et al., J. Org. Chem. (1974) 39:192, which are incorporated herein by reference. See, also, Calbiochem Catalog, (San Diego, CA, 1989), p. 244-247. More preferably, the photosensitive group will be a nitro benzylic compound, such as o-nitrobenzyl or benzylsulfonyl groups. In a preferred embodiment, 6-nitroveratryloxycarbonyl (NVOC) and its derivatives, such as and 6-nitropiperonyloxycarbonyl (NPOC), $\alpha,\alpha$-dimethyl-dimethoxybenzyloxycarbonyl (DDZ) or 1-pyrenylmethyl may be employed.

When the selected binding member is biotin or a biotin analog, photosensitive protecting groups may be provided at the N-1', N-3' or to an oxygen, imino, or sulfur group at the 2'-C position of the ureido ring. When the protecting group is attached to a 2'-C-O- position, the group will preferably be an o-nitro benzylic group having a hydrogen atom at the alpha benzylic position. In such case, the biotin or biotin residue is an imidazolidine group. When the protecting group is attached to a 1'-N or 3'-N atom, the protecting group will preferably be an o-nitro benzylic group having a hydrogen atom bound to the alpha carbon atom and optionally an oxycarbonyl group linking the alpha carbon atom through the oxygen atom. In the latter case, the derivatized nitrogen atom of the imidazolidone group will be bound to the carbon atom of the oxycarbonyl group.

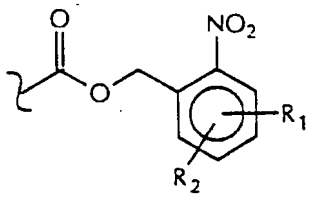
A preferred embodiment of the invention has the following formula:



where X and Z are hydrogen, or oxycarbonyls of lower alkyl, aryl, or benzyl groups, provided that X and Z are not both hydrogen; R is hydrogen, lower alkyl, aryl, carboxylate, alkyl formate, aryl formate, formamide, N-alkylformamide, N-succinimidyl, hydroxyl, alkoxyl, thiol, thioether, disulfide, hydrazide or an amine group; U is O, S, or NH; Y is sulfur, oxygen, methylene, carbonyl, or a sulfinyl, or sulfonyl group, or Y represents two hydrogen atoms attached to the respective carbons; and n = 0-7. Also, inorganic and organic acid addition salts of the above compounds can be employed. Furthermore, R can represent a surface or a surface provided with a suitable crosslinking group. In another preferred embodiment, R is hydrogen, lower alkyl, aryl, carboxylate, alkyl formate, aryl formate, formamide, N-alkylformamide, N-succinimidyl, hydroxyl, alkoxyl, thiol, thioether, disulfide, hydrazide or an amine group connected to a linking group of a suitable length, such as monomer, dimer, trimer, or oligomer of 6-aminocaproic acid, an oligomer of ethylene glycol having up to 10 units dioxadodecane-propyl, or other suitable linkers. A more preferred embodiment is when R is methyl formate or p-nitrophenyl formate. A further preferred embodiment is when U is O, Y is S, and n=4. A preferred embodiment is when Y represents two hydrogen atoms attached to respective carbons, which eliminates the lower ring leaving a methyl group and a hexanoic acid group:



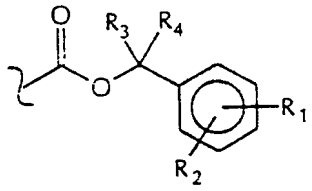
A further preferred embodiment is when X or Z is a nitro aromatic compound containing a benzylic hydrogen ortho to the nitro group. A still further preferred embodiment is when X or Z has the formula:



where $R_1$ and $R_2$ are hydrogen, lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, sulfonate, formamido or phosphido groups. A further preferred embodiment is when X or Z is a nitroveratryloxycarbonyl group.

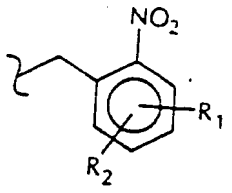
Most preferred embodiments are when X is 6-nitroveratryloxycarbonyl, Z is hydrogen, and R is methyl formate or p-nitrophenyl formate.

Another preferred embodiment is when X or Z is a ring-disubstituted benzyloxycarbonyl group having the formula:



where $R_1$ and $R_2$ are hydrogen, lower alkyl, aryl, benzyl, pyrenyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido or phosphido groups, and $R_3$ and $R_4$ are hydrogen, lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido or phosphido groups. More preferably $R_1$ and $R_2$ are methoxy groups. More preferably $R_3$ and $R_4$ are methyl groups. A most preferred embodiment is when $R_1$ and $R_2$ are methoxy groups and $R_3$ and $R_4$ are methyl groups.

A further preferred embodiment is when X has the formula:

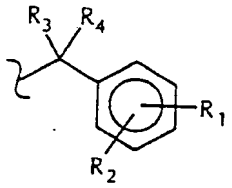


where $R_1$ and $R_2$ are hydrogen, lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido or phosphido groups.

A still further preferred embodiment is when X is a nitroveratryl group.

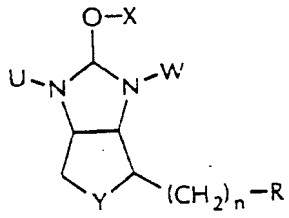
Most preferred embodiments are when X is 6-nitroveratryl, U or W is hydrogen, and R is methyl formate or p-nitrophenyl formate.

A further preferred embodiment is when X is a ring-disubstituted benzyl group. A more preferred embodiment is when the ring-disubstituted benzyl group has the formula:



where $R_1$, $R_2$, $R_3$, and $R_4$ are hydrogen, lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido or phosphido groups. Most preferred embodiments are when $R_1$ and $R_2$ are methoxy and $R_3$ and $R_4$ are methyl.

Another preferred embodiment is when the composition has the following formula:



where X is hydrogen, lower alkyl, aryl, or benzyl; U and W are hydrogen, lower alkyl, aryl, or benzyl groups, provided that only one of U and W is present; R is hydrogen, lower alkyl, aryl, carboxylate, alkyl formate, aryl formate, formamide, N-alkyl formamide, N-succinimidyl, hydroxyl, alkoxyl, thiol, thioether, disulfide, hydrazide, or amine groups; Y is sulfur, oxygen, methylene, carbonyl, or a sulfinyl, or sulfonyl group, or Y represents two hydrogen atoms attached to the respective carbons; and n = 0-7. Also, inorganic and organic acid addition salts of the above compounds are suitable. A more preferred embodiment is when Y is sulfur and n = 4.

Clearly, many photosensitive protecting groups are suitable for use in the present inventive methods. Some examples of acceptable photosensitive protecting groups are presented in Table 3. Also, some example protecting groups and their corresponding wavelengths for deprotection are provided in Table 4.

Table 3

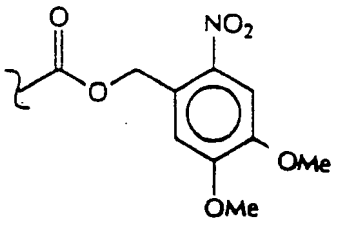
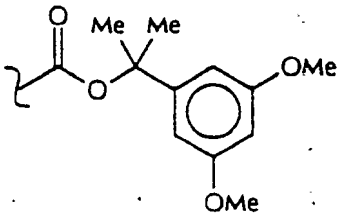
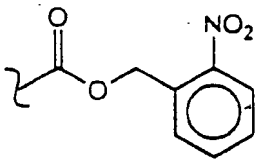
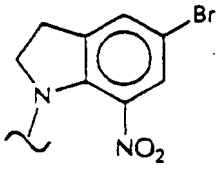
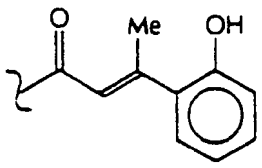
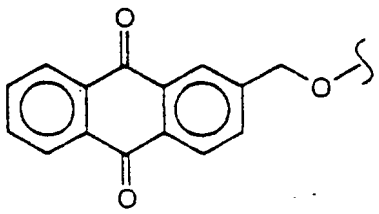
| Example Protecting Groups | Name |
|---|---|
|  | 6-nitroveratryloxy-carbonyl (NVOC) |
|  | dimethyldimethoxy-benzyloxycarbonyl (DDZ) |
|  | nitrobenzyloxycarbonyl (NBOC) |
|  | 5-bromo-7-nitroindolinyl (BNI) |
|  | O-hydroxy-α-methyl-cinnamoyl (HMC) |
|  | 2-oxymethylene anthra-quinone (OMA) |

Table 4:

| Example protecting groups and their deprotection wavelengths | |
|---|---|
| Group | Deprotection Wavelength |
| Nitroveratryloxycarbonyl | UV (300-350 nm) |
| Nitrobenzyloxycarbonyl | UV (300-350 nm) |
| Dimethyldimethoxybenzyloxycarbonyl | UV (280-300 nm) |
| 5-Bromo-7-nitroindolinyl | UV (420 nm) |
| o-Hydroxy-α-methyl cinnamoyl | UV (300-350 nm) |
| 2-Oxymethylene anthraquinone | UV (350 nm) |

B. Irradiation

Once the surface is covered with a plurality of caged binding members, selected regions of the surface may be irradiated to provide activated binding members. Predefined regions of the surface may be selectively activated by electron beam lithography, ion beam lithography, X-ray lithography, or any other radiation method. In a preferred embodiment, the radiation is UV, near IR, or visible light. The light source may be coherent or noncoherent. The protective group may alternatively be an electrochemically-sensitive group which may be removed in the presence of an electric current.

In some embodiments, the exposed area is less than about 1 $cm^2$ or less than about 1 $mm^2$. In preferred embodiments the exposed area is less than about 10,000 $\mu m^2$ or, more preferably, less than about 100 $\mu m^2$. Spaces between activated regions are not critical and will generally be greater than about 1 $\mu m$.

When photoactivatable binding members are used, they are preferably exposed to light through a suitable mask using photolithographic techniques well known in the semiconductor industry and described in, for example, Sze, VLSI Technology, McGraw-Hill (1983), which is incorporated herein by reference. In one embodiment, the mask is a transparent support material coated with a layer of opaque material. Portions of the opaque material are removed, leaving opaque material in the precise pattern desired on the substrate surface. The mask is brought into close proximity with or directly into contact with the surface. Openings in the mask correspond to locations on the surface where it is desired to photoremove protecting groups from the binding members. Alignment may be performed using

conventional alignment techniques in which alignment marks are used to accurately overlay successive masks with previous patterning steps. Other alignment techniques may be used, for example, interferometric techniques such as the one described in Flanders, et al., "A New Interferometric Alignment Technique," App. Phys. Lett. (1977) 31:426-428, which is incorporated herein by reference.

To enhance contrast of light applied to the substrate it may be desirable to provide contrast enhancement materials between the mask and the substrate. This contrast enhancement layer may comprise a molecule which is decomposed by light such as quinone diazide.

The light may be from a conventional incandescent source, an arc lamp, a laser, or the like. If noncoherent sources of light are used it may be desirable to provide a thick- or multi-layered mask to prevent spreading of the light on the substrate. Generally, lasers may be preferable because they can more easily provide wavelengths particularly suited for a chromophore of the photosensitive group.

While the invention is illustrated primarily herein by way of the use of a mask to illuminate the substrate, other techniques may also be used. For example, the substrate may be rotated under a modulated laser or diode light source. Such techniques are discussed in, for example, U.S. Patent No. 4,719,615, which is incorporated herein by reference.

The substrate may be irradiated either in contact with or not in contact with a solution and, preferably, is irradiated in contact with the solution. The solution may contain reagents to prevent by-products of irradiation from interfering with subsequent binding reactions. Such by-products might include, for example, carbon dioxide, nitrosocarbonyl compounds, styrene derivatives, indole derivatives, and products of their photochemical reactions. Reagents added to the solution may include, for example, acidic or basic buffers, thiols, substituted hydrazines and hydroxylamines, reducing agents (e.g., NADH or bisulfite ion) or reagents known to react with a given functional group (e.g., aryl nitroso + glyoxylic acid → aryl formhydroxamate + $CO_2$).

Preferably, however, protecting groups will be selected which do not cause significant interferences with the binding reactions. Also, wash steps will be incorporated so that the by-products do not interfere with the reactions.

In a preferred embodiment, a surface provided with a plurality of sites occupied by photosensitive N-derivatives of biotin or biotin analogs is exposed to a desired light pattern to cause loss of some or all of the photosensitive protecting groups at predefined regions on the surface. Such irradiation of the N-derivatized biotin compounds of the present invention leads to formation of surface-bound biotin or biotin analogs having a strong specific binding affinity for avidin or avidin analogs. The specific binding affinity of biotin and avidin is one of the strongest known between macromolecules ($K_a = 10^{15}$ $M^{-1}$). This binding persists when the carboxyl terminus of biotin is attached to another entity, e.g., a surface, or when avidin is attached to another molecule. Avidin possesses four subunits having specific binding affinity for biotin molecules. For example, deprotected biotin sites may be incubated with avidin or an avidin conjugate of an anti-ligand, e.g., an antibody, to provide a localized concentration of the desired anti-ligand on the surface. When incubation with avidin alone is performed, it is necessary to further incubate the resulting product with a preselected species having specific binding affinity for avidin, e.g., a biotinylated anti-ligand. Thus, biotinylated anti-ligands can be bound to the free sites of avidin to afford anti-ligands immobilized at predefined regions on the surface. For a general discussion of the use of the biotin-avidin interaction in molecular biology, see Bayer, et al. Once localization of the anti-ligand is complete, the light pattern can be changed and the same or a different anti-ligand can be localized at other discrete sites on the surface.

V. Attachment of Anti-ligands

An anti-ligand is one or more molecules that recognize a particular ligand in solution. Examples of ligands that can be investigated by this invention include, but are not

restricted to agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, antigenic determinants, hormones, hormone receptors, steroids, peptides, enzymes, substrates, cofactors, drugs, lectins, sugars, oligonucleotides, oligosaccharides, proteins, and monoclonal and polyclonal antibodies.

Anti-ligands that mediate a biological function on binding with particular ligand(s) are of most interest. Suitable anti-ligands include relatively small, single molecules, such as cofactors, which show specific binding properties. Typically, anti-ligands will be greater than about 100 daltons in size and more typically will be greater than about 1kD in size. Other examples of anti-ligands include, but are not restricted to, the common class of receptors associated with the surface membrane of cells and include, for instance, the immunologically important receptors of B-cells, T-cells, macrophages and the like. Other examples of anti-ligands that can be investigated by this invention include but are not restricted to hormone receptors, hormones, drugs, cellular receptors, membrane transport proteins, steroids, peptides, enzymes, substrates, cofactors, vitamins, lectins, sugars, oligonucleotides, oligosaccharides, viral epitopes, antigenic determinants, glycoproteins, and immunoglobulins, e.g., monoclonal and polyclonal antibodies.

In a preferred embodiment, the anti-ligand will be a biotinylated receptor which binds specifically to avidin. Many biotinylated anti-ligands and biotinylating reagents are commercially available. (See, for example, Vector Laboratories, Inc., Catalog, Burlingame, CA) Methods for biotinylating desired anti-ligands are well-known in the art and are described, for example, at Bayer, et al.

In a preferred embodiment a plurality of anti-ligands is immobilized on a surface by first attaching photoreactive caged binding members to the surface. The caged binding members on a predefined region of the surface are exposed to light to give binding members having a high affinity for a specific binding substance. The activated binding members on the predefined region are then incubated with the specific

binding substance, the surface is washed free of unbound specific binding substance, and the surface is incubated with a desired anti-ligand or anti-ligand conjugate. The exact incubation conditions, e.g., time, temperature, pH, will depend upon the species used and will be readily apparent to one skilled in the art. After washing the surface free of unbound anti-ligand, the above steps can be repeated on a different region of the surface.

In another embodiment of the invention a plurality of anti-ligands is immobilized on a surface as described above, except the attachment of anti-ligands to specific binding substance is carried out prior to introducing the specific binding substance to the surface.

In a further embodiment the anti-ligand is a monoclonal or polyclonal antibody. In a still further preferred embodiment the anti-ligand is a biotinylated antibody or biotinylated receptor.

A most preferred embodiment of the invention is when the binding member is biotin or a biotin analog and the specific binding substance is avidin or an avidin analog.

VI. Screenings and Assays

A surface prepared according to the methods described above can be used to screen for ligands having high affinity for immobilized anti-ligands. Screening can be performed by immobilizing a plurality of anti-ligands on predefined regions of a surface by the methods described above. A solution containing a marked (labelled) ligand is introduced to the surface and incubated for a suitable period of time. The surface is then washed free of unbound ligand and the anti-ligands having high affinity for the ligand are identified by identifying those regions on the surface where markers are located. Suitable markers include, but are not limited to, radiolabels, chromophores, fluorophores, chemiluminescent moieties, and transition metals. Alternatively, the presence of ligands may be detected using a variety of other techniques, such as an assay with a labelled enzyme, antibody, and the like. Other techniques using various marker systems for

detecting bound ligand will be readily apparent to those skilled in the art.

In a preferred embodiment, a substrate prepared as discussed above can be exposed to a solution containing marked ligand such as a marked antigen. The ligand can be marked in any of a variety of ways, but in one embodiment marking is effected with a radioactive label. The marked antigen binds with high affinity to an immobilized antibody previously localized on the surface. After washing the surface free of unbound ligand, the surface is placed proximate to x-ray film to identify the antibodies that recognize the antigen. Alternatively, a fluorescent marker may be provided and detection may be by way of a charge-coupled device (CCD), fluorescence microscopy or laser scanning.

When autoradiography is the detection method used, the marker is a radioactive label, such as $^{32}P$. The marker on the surface is exposed to X-ray film, which is developed and read out on a scanner. An exposure time of about 1 hour is typical in one embodiment. Fluorescence detection using a fluorophore label, such as fluorescein, attached to the ligand will usually require shorter exposure times.

Quantitative assays for ligand concentrations can also be performed according to the present invention. In a direct assay method, the surface containing localized anti-ligands prepared as described above, is incubated with a solution containing a marked ligand for a suitable period of time. The surface is then washed free of unbound ligand. The amount of marker present at predefined regions of the surface is then measured and can be related to the amount of ligand in solution. Methods and conditions for performing such assays are well-known and are presented at, for example, L. Hood, et al., Immunology, Benjamin/Cummings (1978) and E. Harlow, et al., Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory, (1988). See, also U.S. Patent No. 4,376,110 for methods of performing sandwich assays. The precise conditions for performing these steps will be apparent to one skilled in the art.

A competitive assay method can also be employed by the present invention. Such a method involves immobilizing anti-ligands on predefined regions of a surface as described above. An unmarked ligand is then bound to anti-ligands on the surface having specific binding affinity for the ligand. A solution containing marked ligand is then introduced to the surface and incubated for a suitable time. The surface is then washed free of unbound reagents and an amount of marker remaining on the surface is measured. Alternatively, marked and unmarked ligand can be exposed to the surface simultaneously. The amount of marker remaining on predefined regions of the surface can be related to the amount of unknown ligand in solution.

Use of the invention herein is illustrated primarily with reference to screenings of ligands for anti-ligands and assays for ligands. The invention will, however, find many other uses. For example, the invention may be used in information storage (e.g., on optical disks), production of molecular electronic devices, production of stationary phases in separation sciences, and in immobilization of cells, proteins, lectins, nucleic acids, polysaccharides and the like in any desired pattern on a surface via molecular recognition of a specific anti-ligand.

The invention has been described primarily with reference to the use of photoremovable protecting groups, but it will be readily recognized by those of skill in the art that other types of groups can be used and that other sources of radiation can also be used. For example, in some embodiments it may be desirable to use protecting groups sensitive to electron beam irradiation, X-ray irradiation, X-ray lithography, or combinations thereof. Alternatively, the group could be removed by exposure to an electric current.

It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with

reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

VII. Examples

The following examples of preferred embodiments of the present invention are presented by way of illustration only and do not suggest that the above- described methods and compositions are in any way limited by the specific examples set forth below.

Synthesis of Photoreactive N-1'-Derivatives of Biotin

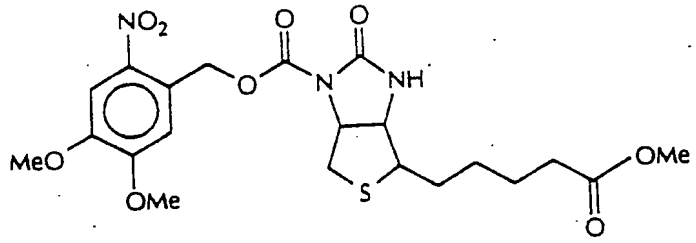
Methods for the preparation of acyl imidazolinones, such as biotin derivatives, are well-known. See, for example, Kohn, et al., J. Org. Chem. (1977), 42, 941-948 and Knappe, et al., Biochem. Z. (1961), 335, 168-176. Treatment of biotin methyl ester with methyl chloroformate in refluxing chloroform (no base) for 72-80 h afforded a mixture heavily favoring the N-1'-derivative. Under similar conditions, the use of nitroveratryloxycarbonyl (NVOC) chloride (Amit, et al., J. Org. Chem. (1974), 39, 192-196) gave N-1'-(nitroveratryloxycarbonyl)-biotin methyl ester (NVOC-biotin-OMe) in 47% yield after chromatography and crystallization. Likewise, N-1'-(nitroveratryloxycarbonyl)-biotin p-nitrophenyl ester (NVOC-biotin-ONP) was obtained in 39% yield.

The structural assignment is based on precedent as well as spectroscopic properties. See, for example, E. Becker, High Resolution NMR, 2nd ed., Acad. Press (1980). In particular, the 1H NMR spectrum readily differentiates the ring fusion protons bearing 1) a urea nitrogen (ca 4.2 ppm) and 2) an imide nitrogen (ca 4.8 ppm). Through the use of COSY (Derome, Modern NMR Techniques for Chemistry Research, Pergamon Press, Oxford (1987)) on N-1'-(nitroveratryloxycarbonyl)-biotin p-nitrophenyl ester, it was determined that the former ring fusion proton is vicinal to a methine adjacent to sulfur, and that the latter is vicinal to a methylene adjacent to sulfur.

Using the conditions reported by Kohn, et al. biotin methyl ester is similarly derivatized with

dimethyldimethoxycarbobenzyloxycarbonyl (DDZ) chloride or 1-pyrenylmethyloxycarbonyl (PYROC) chloride to afford the photolabile compounds N-1'-(dimethyldimethoxycarbobenzyloxycarbonyl) biotin methyl ester (DDZ-biotin-OMe) and N-1'-(1-pyrenylmethyloxycarbonyl) biotin methyl ester (PYROL-biotin-OMe), respectively. The methods of Knappe, et al., and Kohn, et al., can be employed to prepare analogous compounds and are incorporated by reference herein.

Example A: Preparation of NVOC-biotin-OMe (N-1'-(6-nitroveratryloxycarbonyl)-biotin methyl ester)




2.00 g (8.19 mmol) of D-biotin was added to a methanolic HCl solution prepared from 2.5 ml of acetyl chloride in 40 ml of anhydrous methanol. After stirring for 15 hours, the solvent was removed under reduced pressure to afford 2.11 g of the product biotin-OMe (biotin methyl ester) as a white solid, MP 116-118° C (100% yield).

NVOC-biotin-OMe was prepared from biotin via the intermediate, biotin methyl ester (biotin-OMe) by either of two methods.

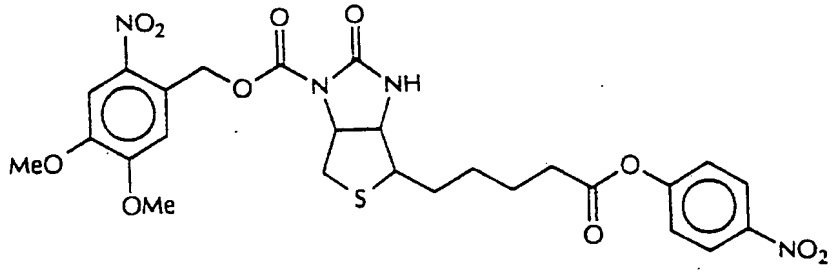
1. A solution of 1.00 g (3.87 mmol) of biotin-OMe and 1.60 g (5.81 mmol) of 6-nitroveratryloxycarbonyl chloride in 10 ml of chloroform was heated to reflux for 50 hours. The product was purified via flash-column chromatography on silica gel (3% methanol, 3% acetone, 94% chloroform as eluent) to afford 0.90 g of NVOC-biotin-OMe as a yellow solid (47% yield, 84% yield based on unreacted starting biotin-OMe), MP

199-203° C, and 0.44 g of recovered biotin-OMe (44% recovery). The product, NVOC-biotin-OMe, was recrystallized from methylene chloride/ether.

2. A solution of 3.4 g (12 mmol) of 6-nitroveratryloxycarbonyl chloride in 40 ml of methylene chloride was added to a solution of 1.1 ml (14 mmol) of pyridine and 1.3 g (13 mmol) of phenol in 15 ml of methylene chloride cooled to 0° C. The reaction mixture was allowed to warm to room temperature and was stirred for 19 hours. The solution was partitioned between methylene chloride and 1 N HCl, the organic phase was separated and dried with magnesium sulfate, and the solvent was removed under reduced pressure to give 4.1 g of a brown oil. Purification via flash-column chromatography on silica gel (90% methylene chloride/10% hexane as the eluent) afforded 2.0 g of the product, 6-nitroveratryl phenyl carbonate, as a colorless oil.

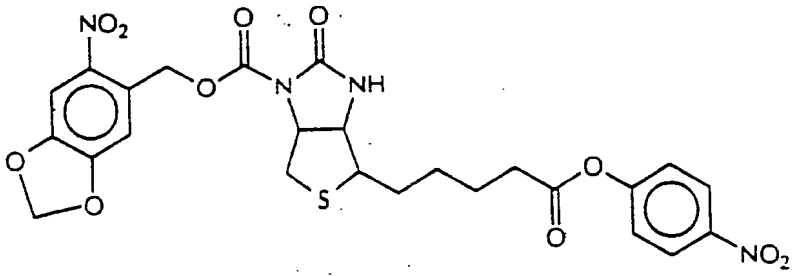
A mixture of 399 mg (1.20 mmol) of 6-nitroveratryl phenyl carbonate in 5 ml of chloroform and 202 mg (0.782 mmol) of biotin-OMe were heated to reflux. After 50 hours TLC showed that no reaction occurred, therefore 35 mg of 60% sodium hydride (0.88 mmol) was added in two equal portions over 15 minutes. After an additional 16 hours at reflux, the reaction was quenched with 3 drops of glacial acetic acid. The product was purified via flash-column chromatography on silica gel (3% methanol, 3% acetone, 94% chloroform as the eluent) to afford 264 mg of the product, NVOC-biotin-OMe, as an off-white solid (68% yield, 75% yield based on recovered unreacted biotin-OMe) and 20 mg of recovered biotin-OMe (10% yield).

Example B: Preparation of NVOC-biotin-ONP (N-1'-(6-nitroveratryloxycarbonyl)-biotin para-nitrophenyl ester)



A mixture of 0.340 g (0.930 mmol) of biotin p-nitrophenyl ester (purchased from Sigma Chemical Co., St. Louis) and 0.450 g (1.63 mmol) of 6-nitroveratryloxycarbonyl chloride in 4 ml of chloroform was heated to reflux for 65 hours. The mixture was purified via flash-column chromatography on silica gel (3% methanol, 3% acetone, 94% chloroform as the eluent) to produce 0.231 g of NVOC-biotin-ONP as a beige solid (39% yield, 93% yield based on unreacted biotin-ONP), MP 203-205° C, and 0.21 g of recovered unreacted biotin-ONP (58% yield). The product was further purified via recrystallization from chloroform/hexane.

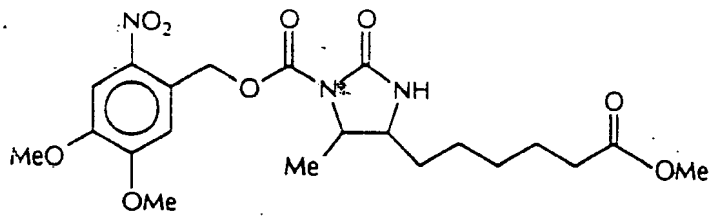
Example C: Preparation of NPOC-biotin-ONP (N-1'-(6-nitropiperonyloxycarbonyl)-biotin para-nitrophenyl ester)



A mixture of 0.365 g (1.00 mmol) of biotin p-nitrophenyl ester and 0.410 g (1.58 mmol) of 6-

nitropiperonyloxycarbonyl chloride in 5 ml of chloroform was heated at reflux for 62 hours. The product was purified via flash-column chromatography on silica gel (3% methanol, 3% acetone, 94% chloroform as the eluent) to produce 0.231 g of product as a beige solid (39% yield, 93% based on unrecovered starting material), MP 203-205° C, and 0.21 g of recovered biotin-ONP (58% yield). As described, the product was further purified via recrystallization from chloroform/hexane.

Example D: Preparation of NVOC-DT-biotin-OMe (N-1'-(6-nitroveratryloxycarbonyl)-dethiobiotin methyl ester)

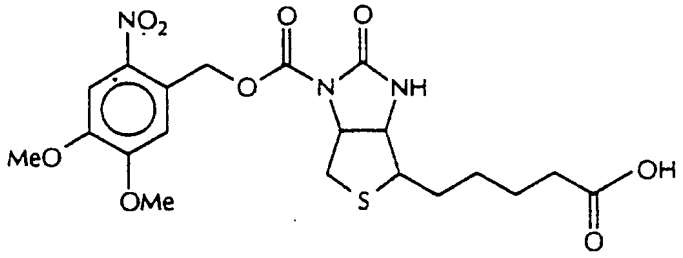



A slurry of 50 mg (0.19 mmol) of biotin-OMe and about 1 g of Raney Nickel active catalyst (50% solution in $H_2O$ from Aldrich Chemical Co.) in 4 ml of methanol was stirred at room temperature for one hour. The reaction mixture was diluted with chloroform, filtered and the recovered catalyst was washed with methanol. The combined wash and filtrate were partitioned between chloroform and saturated sodium chloride acidified to pH 2 with 1 N HCl. The combined organic phases were dried over magnesium sulfate, and the solvent removed under reduced pressure to give 34 mg of pure DT-biotin-OMe as a white solid, MP 68-72° C (77% yield).

A mixture of 34 mg (0.15 mmol) of DT-biotin methyl ester and 74 mg (0.27 mmol) of 6-nitroveratryloxycarbonyl chloride in 3 ml of chloroform was heated to reflux for 15 hours. The products are purified via flash-column chromatography on silica gel (3% methanol, 97% chloroform as

the eluent) to afford 45 mg of a 1:3 mixture of products as a yellow solid (65% yield), MP 152-156° C.

Example E: Preparation of NVOC-biotin-OH (N-1'-(6-nitroveratryloxycarbonyl)-biotin)




A solution of 262 mg (0.527 mmol) of NVOC-biotin-OME, prepared in by either method of Example A, in 15 ml of tetrahydrofuran and 2 ml of dimethylforamide was treated with 10 ml of 1 N HCl. The reaction mixture was heated to reflux for 49 hours, cooled to room temperature, and the solvent removed under reduced pressure. The crude product was purified via flash-column chromatography on silica gel (10% methanol, 90% chloroform as the eluent) to afford 178 mg of the pure product, NVOC-biotin-OH, as a white solid (70% yield), MP 219-223° C.

Example F: Chromatographic Evidence for Photoremoval of the NVOC group from NVOC-biotin-ONP

A 100 mM solution of NVOC-biotin-ONP in acetonitrile, or biotin in water, was placed in a quartz cuvette with a 2.0 mm pathlength. The cuvette was irradiated for two minutes at a power of 1 watt/cm$^2$ with a 500 W Hg(Xe) arc lamp (Oriel #66142) having a 305 nm long pass filter (Oriel #51450). Illuminated and non-illuminated samples were then subjected to reverse-phase HPLC.

Shown in FIG. 1 are chromatographs of illuminated biotin and illuminated NVOC-biotin-ONP. The results support the following: 1) biotin was unaffected by the illumination;

and 2) NVOC-biotin-ONP was converted to Biotin-ONP by the illumination.

Example G: Estimation of the Affinity of NVOC-biotin-OMe for Avidin Before and After Illumination

All procedures using NVOC-biotin-OMe were conducted in dim red light. NVOC-biotin-OMe was dissolved in dimethylformamide (DMF) to a concentration 1 mM and diluted to 100 μM with phosphate-buffered saline pH 7.4. In order to remove any contaminants having high affinity for avidin, 2 ml of the NVOC-biotin-OMe solution was mixed with 1 ml of packed streptavidin-Sepharose-4B resin (SIGMA) which had been washed thoroughly to remove any non-covalently bound avidin. After stirring for three hours, the resin was removed by centrifugation followed by filtration (0.2 micron nylon filter). The concentration of the NVOC-biotin-OMe solution (measured by 350 nm absorbance) was not significantly reduced by the resin treatment.

Microtiter wells (Beckman EIA plates) were treated for 1 hr with 200 μl of 0.5 μg/ml of streptavidin in 10 mM sodium bicarbonate buffer (pH 9.6). After removal of the streptavidin solution and washing with phosphate buffered saline (PBS)/0.05% Tween 20, the wells were incubated for 1 hr at room temperature with 200 μl of PBS containing various concentrations of illuminated (as detailed above) and non-illuminated biotin or streptavidin-Sepharose treated NVOC-biotin-OMe. The wells were then washed with PBS/Tween 20 and incubated for 1 hr at room temperature with 200 μl of PBS containing $^{3}$H-biotin (30 Ci/mmol, New England Nuclear). The wells were then washed with PBS/Tween and treated for 30 minutes at room temperature with 200 μl of 10% trichloroacetic acid in water. The radioactivity in 100 μl was then determined by liquid scintillation counting.

The results of a representative binding experiment are shown in FIG. 2. The experiment was done three times with similar results. The data indicate that NVOC-biotin-OMe has very low affinity for avidin as indicated by the fact that pre-incubation of avidin with concentrations of NVOC-biotin-OMe

("caged biotin") as high as $10^{-5}$ M had no significant effect on the subsequent binding of $^{3}$H-biotin. In addition, the results indicate that illumination of NVOC-biotin-OMe generates a biotin derivative ("IL caged biotin") that was nearly as effective as biotin in blocking the subsequent binding of $^{3}$H-biotin. Combined with the chromatographic evidence above, the data indicate that illumination of NVOC derivatives of biotin leads to removal of the NVOC group.

Example H: Demonstration of Photoremoval of NVOC group from NVOC-Biotin Attached to a Membrane

Nitrocellulose membrane filters (Biorad) were reacted with 5% bovine serum albumin in Tris-buffered saline (TBS) for 3 hr at room temperature. The membranes were washed with TBS, cut into 1 $cm^2$ sections and then reacted for 3 hr at room temperature (in the dark) with 10% DMSO/100 mM sodium borate buffer (pH 8.6) alone, 10 mM of biotin-N-hydroxysuccinimidyl ester in 10% DMSO/100 mM sodium borate buffer (pH 8.6), or NVOC-biotin-ONP in 50% DMSO/100 mM sodium borate buffer (pH 8.6). After washing with TBS, half the sections that were treated with NVOC-biotin-ONP were illuminated in a manner identical to that described above. After washing with TBS, the membrane sections were incubated for 1 hr at room temperature with TBS containing 0.1% bovine serum albumin and 0.1 $\mu$Ci/ml $^{125}$I-streptavidin (Amersham). After washing with TBS, radioactivity on the membrane section was quantitated by gamma counting.

FIG. 3 shows the data for a representative experiment. Binding of $^{125}$I-streptavidin was approximately 3-fold higher with biotinylated membranes than with control membranes. Binding of $^{125}$I-streptavidin to non-illuminated NVOC-biotinylated membranes was not significantly different from non-biotinylated control membranes. $^{125}$I-streptavidin binding to illuminated NVOC-biotinylated membranes was approximately equal to that of biotinylated membranes. These data indicate that membrane-bound NVOC-biotin has low affinity for streptavidin and that illumination greatly increases

streptavidin binding by removing the NVOC group from the biotin group.

Immobilization of anti-ligands on solid supports

Example I: Preparation of Caged-Biotin Glass Plates

Commercially available glass microscope slides were derivatized with N-BOC-aminopropyltriethoxy silane according to literature procedures (for example, see J. Chromatography, 1974, Vol. 97, p. 33). The slides were incubated in a solution of 20% trifluoroacetic acid in methylene chloride for 30 minutes to remove the BOC protecting group. After washing sequentially with methylene chloride, dimethylformamide, and ethanol, the slides were neutralized by immersing in a solution of 10% diisopropylethyl amine in methylene chloride for 30 minutes and further washed with methylene chloride.

N-BOC-6-aminocaproic acid was converted to the BOP-activated ester in preparation for reaction with the derivatized glass slide. A solution of a 197 mg (0.445 mmol) of benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) in 0.40 ml of dimethylformamide was added to a solution of 86 mg (0.37 mmol) of N-BOC-6-aminocaproic acid, 56 mg (0.4 mmol) of 1-hydroxybenzotriazole hydrate (HOBT), and 0.14 ml (0.80 mmol) of diisopropylethyl amine in 0.40 ml of dimethylformamide. After 10 minutes the resultant solution was diluted with 3.20 ml of dimethylformamide to give a 0.10 M solution of the activated ester. The derivatized slides were incubated with 0.5 ml of the activated ester solution and after two hours of coupling, were sequentially washed with dimethylformamide, ethanol and methylene chloride. The BOC protecting groups were removed from the aminocaproic acid moieties, and the slide was subsequentially washed as described above. Biotin derivatives were coupled to the glass plates by either of two methods, as illustrated for NVOC-biotin.

Method A (via the BOP ester)

The BOP derivative of NVOC-biotin-OH was prepared by adding a solution of 40 mg (0.099 mmol) of BOP in 0.09 ml of

dimethylsulfoxide to a solution of 43 mg (0.090 mmol) of NVOC-biotin-OH, 13 mg (0.096 mmol) of HOBT, and 0.035 ml of diisopropylethyl amine in 0.090 ml of dimethylsulfoxide. After five minutes, the resulting solid was diluted to 1.80 ml with dimethylsulfoxide to give a 0.05 M solution of the activated ester. Approximately 0.5 ml of the activated ester solution was applied to the surface of the derivatized glass surface. After being exposed to the activated ester for two hours, the slides were washed sequentially with dimethylformamide, ethanol, and methylene chloride to yield a NVOC-biotin-caproic-propyl derivatized surface.

Method B (via the ONP ester)

Approximately 0.5 ml of an 0.10 M solution of NVOC-biotin-ONP in dimethylformamide was applied to the surface of the derivatized glass slide. After 2-24 hours, the slides were washed sequentially with dimethylformamide, ethanol, and methylene chloride to yield a NVOC-biotin-caproic-propyl derivatized surface.

Example J: Photodeprotection of an NVOC-Biotin-Caproic-Propyl-derivatized slide, and subsequent labeling with a Fluorescein-Streptavidin conjugate

A glass microscope slide to which NVOC-biotin has been covalently attached via a caproic-propyl spacer, as described in the Example I, was mounted on a custom flow cell and illuminated through a 500 $\mu$m x 500 $\mu$m checkerboard-pattern mask (Photo Sciences Inc., Torrance, CA) using broad-band UV/blue light. The light source was a 500 W Mercury arc lamp (Oriel Model 87330) equipped with a 350 nm - 450 nm dichroic reflector and produced actinic light having an intensity of 12 mW/cm$^2$ as measured through a 360 nm bandpass filter. The derivatized surface was photolyzed in flowing dioxane for 15 minutes, removed from the flow cell, and sequentially rinsed in deionized water, ethanol, and methylene chloride.

After incubation for one hour in a solution containing filtered PBS, 1% BSA, 0.05% Tween 20, pH 7.4, the activated surface was treated with a solution containing a Fluorescein-derivative of Streptavidin (Molecular Probes; 10 μg/ml in PBS/BSA/Tween 20) for one hour at room temperature. The slide was vortexed twice in PBS, 0.05% Tween 20, pH 7.4 for 10 minutes, rinsed with deionized water, and allowed to dry. The slide was examined with a scanning fluorescence microscope (Zeiss Axioskop equipped with a Newport Model PM500-C motion controller, a Spectra-Physics Model 2020 argon-ion laser producing a 488 nm excitation light; and a 520 nm long-pass emission filter) interfaced with a photon-counting device (Hamamatsu Model 9403-02 photomultiplier; Stanford Research Systems Model SR445 amplifier and Model SR430 multichannel scaler, IBM compatible PC) to generate a two-dimensional image consisting of fluorescence intensity data as a function of x,y position. An example of this technique is described in U.S. Patent Applications Serial Numbers 07/362,901 and 07/492,462, filed June 7, 1989 and March 7, 1990, respectively, and incorporated herein by reference. FIG. 4 shows an example of the images obtained.

The light squares indicate regions of high fluorescence intensity resulting from localization of the fluorescein label attached to the anti-ligand. This experiment demonstrates enhanced binding of streptavidin upon photodeprotection of NVOC-biotin coupled to a surface with a caproic-propyl spacer, and spatially-addressable immobilization of an anti-ligand, such as streptavidin, by non-covalent means.

Example K: Photodeprotection of an NVOC-Biotin-Caproic-Propyl-derivatized slide, treatment with Streptavidin, and labeling with a Fluorescein-Biotin conjugate

The microscope slide of Example I having NVOC-biotin covalently attached via a caproic-propyl spacer was illuminated and processed as in Example J, except that after preincubation with the PBS/BSA/Tween 20 solution, the surface was treated with 10 μg/ml solution of Streptavidin (in PBS/BSA/Tween 20)

for 40 minutes at room temperature, followed by incubation with a 1 μM solution of Fluorescein-Biotin (5-(N-((5-(N-(6-(biotinoyl)amino) hexanoyl)amino)pentyl)thioureidyl)fluorescein, in PBS, pH 7.4) for 20 minutes. The resulting slide was then washed, dried, and examined using a scanning fluorescence microscope as described above. FIG. 5 shows an example of the images obtained.

The light squares indicate regions of high fluorescence intensity resulting from localization of the fluorescein label attached to ligand, biotin. This experiment demonstrates the binding of a ligand (a Fluorescein-Biotin complex) to streptavidin immobilized in a spatially-addressable manner.

Example L: Photodeprotection of an NVOC-Biotin-Polyether-derivatized slide and labeling with a Fluorescein-Streptavidin conjugate

A microscope slide to which 3-aminopropyltriethoxy silane had been attached was treated with the BOP-activated ester of 18-amino-6-aza-10,15-dioxa-5-ketooctadecanoic acid using a procedure similar to Example I. The resulting slide was then derivatized with NVOC-Biotin-ONP, and was illuminated, processed, and examined as described in Example J. FIG. 6 shows an example of the images obtained.

This experiment demonstrates spatially-localized streptavidin binding upon photodeprotection of NVOC-Biotin coupled to a surface using an alternative linker, a polyether-glutaric-propyl moiety.

Example M: Photodeprotection of an NPOC-Biotin-Caproic-Propyl-derivatized slide and labeling with a Fluorescein-Streptavidin conjugate

The microscope slide to which NPOC-biotin-ONP had been covalently attached via a caproic-propyl spacer was illuminated, processed, and examined as described in Example J. FIG. 7 shows an example of the images obtained.

This experiment demonstrates spatially-localized streptavidin binding upon photodeprotection of caged-biotin using a different protecting group, NPOC.

Example N: Photodeprotection of an NVOC-Biotin-Caproic-Propyl-derivatized slide in aqueous buffer and labeling with a Bodipy-streptavidin conjugate

The microscope slide of Example I having NVOC-Biotin covalently attached via a caproic-propyl spacer was mounted on a custom flow cell and illuminated using the apparatus described in Example J. The derivatized surface was photolyzed in PBS, 1% BSA, 0.1% Tween 20 for 30 minutes at 12 $mW/cm^2$, removed from the flow cell, and processed as described in Example K, except that Bodipy-streptavidin (Molecular Probes, 50 $\mu g/ml$) was used instead of the fluorescein conjugate. FIG. 8 shows an example of the images obtained.

This experiment demonstrates spatially-localized streptavidin binding upon photodeprotection of NVOC-biotin in a different solvent, in this case an aqueous buffer.

Example O: Evaluation of crosslinking groups of different lengths

A microscope slide derivatized with N-Boc-3-aminopropyltriethoxy silane, as in Example I, was functionalized with three different crosslinkers in different locations by selectively coupling 1, 2 or 3 N-Boc-6-aminocaproic acid linkers to the surface via their BOP-activated esters. This generated four distinct and well defined regions on the surface of the slide having zero, one, two and three 6-aminocaproic acid linkers (4, 11, 18 and 25 atom spacers, respectively) between the surface and the terminal amino functionality used to bind to the carboxyl group of the derivatized biotin. A 0.1 M solution of biotin p-nitrophenyl ester in dimethylformamide was subsequently coupled to the slide, and the relative binding affinity of streptavidin to the surface-bound biotin was measured by incubating the slide with fluoresceinated streptavidin and

measuring the fluorescence intensity as in Example J. The measured relative fluorescence was 38, 68, 85 and 100% (normalized to the fluorescence of area having three caproic linkers), respectively, for zero, one two and three caproic linkers, showing that a higher density of streptavidin was bound to an area of the slide derivatized with biotin spaced relatively far from the surface of the slide. FIG. 9 shows the fluorescence of fluoresceinated streptavidin bound to the glass slide derivatized in this experiment.

Example P: Photodeprotection of an NVOC-Biotin-Caproic-Propyl-derivatized slide and subsequent immobilization of antibodies on the derivatized surface.

A microscope slide to which NVOC-Biotin had been covalently attached via a caproic-propyl spacer was mounted on a custom flow cell and illuminated as described in Example J, except that a hand-cut mask consisting of a horizontal stripe (approximately 2 mm wide) was used. After removal from the flow cell and rinsing, the slide was incubated for 30 minutes with PBS, 1% BSA, 0.05% Tween 20, pH 7.4, followed by 30 minutes treatment with Streptavidin (10 μg/ml in PBS/BSA/Tween 20), rinsing with PBS, 0.05% Tween 20, 60 minutes incubation with biotinylated Rabbit IgG (Vector Laboratories; 50 μg/ml in PBS/BSA/Tween 20), and rinsing with PBS/Tween 20. The surface was then "capped" to prevent subsequent streptavidin binding to the multiple biotin moieties on the biotinylated IgG. That is, streptavidin was used to bind free biotin on IgG. This was accomplished by re-treatment with Streptavidin solution, rinsing with PBS/Tween 20, followed by incubation with free biotin (Molecular Probes; 1 mM in PBS/Tween 20; 10 minutes), and a final PBS/Tween 20 rinse. The slide was then remounted on the flow cell, photolyzed for 30 minutes in PBS/Tween 20 using a hand-cut mask consisting of vertical stripes (approximately 2 mm wide), and processed as described above, except that biotinylated Mouse IgG (Vector Laboratories; 50 μg/ml in PBS/BSA/Tween 20; 30 minute incubation) was used, and the "capping" steps were not repeated. The slide was then

rinsed with deionized water, allowed to dry, and beads of silicone gasket compound (Permatex Ultra Blue) were used to partition the slide into three areas. After pre-incubation for 30 minutes with PBS/BSA/Tween 20, the first area was treated with Fluorescein-labeled anti-Rabbit IgG (Vector Laboratories; made in goat; 100 μg/ml in PBS/BSA/Tween 20). The second area was treated with Fluorescein-labeled anti-Mouse IgG (Vector Laboratories; made in horse; 100 μg/ml in PBS/BSA/Tween 20). The third area was incubated with an equimolar mixture of the two secondary antibodies. The slide was then vortexed twice in PBS/Tween 20 for two minutes, rinsed briefly with deionized water, and allowed to dry. The different regions of the slide were examined using the scanning fluorescence microscope described in Example J.

FIG. 10 shows examples of the images obtained from the glass slide derivatized in this experiment. Figure 10a shows the region of the glass slide treated with fluorescein-anti-Rabbit IgG. As expected, the horizontal stripe, which corresponds to the area where biotinylated Rabbit IgG bound, is intensely fluorescent indicating a high density of bound fluorescein-anti-Rabbit IgG. The vertical stripes in this region are faintly visible, which may be due to the slight cross reactivity of the secondary antibodies. FIG. 10b shows the region of the glass treated with Fluorescein-anti-Mouse IgG. Here, the vertical stripes, where Mouse IgG is bound, are fluorescent while the horizontal areas do not fluoresce appreciably. Finally, FIG. 10c shows the region treated with both secondary antibodies (fluorescein-anti-Rabbit and anti-Mouse). In this case both the vertical and horizontal stripes fluoresce, indicating a high surface density fluorescein and, therefore, of the secondary antibodies. This experiment demonstrates the spatially addressable immobilization of two different antibodies on the same surface.

WHAT IS CLAIMED IS:

1. A method for forming predefined regions on a surface of a solid support, the predefined regions capable of selectively immobilizing an anti-ligand, the method comprising:

a) attaching to the surface a caged binding member having a low affinity for the anti-ligand and which member is convertible by irradiation to a binding member capable of immobilizing the anti-ligand by an interaction having an affinity constant of $10^7$ or stronger; and

b) selectively irradiating the predefined regions of the surface to convert the caged binding member in the predefined regions to the binding member.

2. A method as in claim 1 wherein the anti-ligand is attached to the binding member by a specific binding substance.

3. A method as in claim 2 wherein the specific binding substance is avidin or streptavidin.

4. A method as in claim 3 wherein the specific binding substance is egg avidin.

5. A method as in claim 1 wherein the binding member is attached to the surface by a crosslinking group.

6. A method as in claim 1 wherein the binding member is biotin.

7. A method as in claim 1 wherein the anti-ligand is an immunoglobulin.

8. A method as in claim 1 wherein the immunoglobulin is a monoclonal antibody.

9. A method as in claim 2 wherein the binding member binds to a specific binding substance with an affinity constant ($K_a$) of about $10^{15}$ $M^{-1}$.

10. A method as in claim 1 wherein the surface is silica or glass.

11. A method for attaching an anti-ligand to a surface comprising the steps of:

a) linking to the surface a caged biotin molecule having a relatively low affinity for avidin;

b) irradiating caged biotin molecules on predefined regions of the surface to form biotin analogs having high binding affinities for avidin;

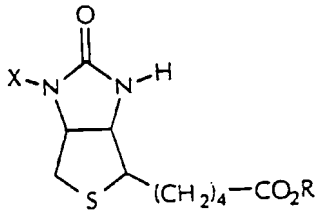
c) reacting the biotin analogs with avidin; and

d) incubating biotinylated anti-ligands with the predefined regions on the surface.

12. A method as in claim 11 wherein the anti-ligand is a monoclonal antibody.

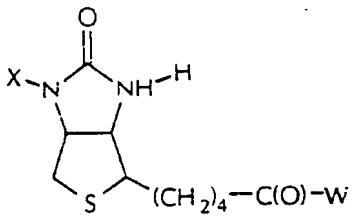
13. A method as in claim 11 wherein the surface is glass or silica.

14. A method as in claim 11 wherein the caged biotin molecule has the formula:



wherein X is selected from the group consisting of oxycarbonyls of lower alkyl, aryl, and benzyl groups, provided that X is not methyl oxycarbonyl; R is selected from the group consisting of hydrogen, lower alkyl, aryl, benzyl, and N-succinimidyl; and acid addition salts of the molecule.

15. A method as in claim 14 wherein the biotin analog has the formula:



wherein W is a surface or a crosslinking group attached to a surface.

16. A method as in claim 11 wherein the biotin analog and avidin have an affinity constant ($K_a$) of about $10^{15}$ $M^{-1}$.

17. A method for localizing an anti-ligand on a surface comprising the steps of:

a) linking to the surface a photoactivatable biotin molecule having low affinity for avidin;

b) exposing the photoactivatable biotin molecule to light to form a biotin analog having a high binding affinity for avidin; and

c) incubating an anti-ligand attached to avidin with the biotin analog.

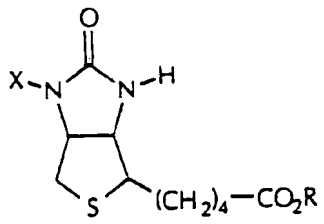
18. A method as in claim 17 wherein the anti-ligand is attached to avidin by a bifunctional crosslinking group.

19. A method as in claim 17 wherein the anti-ligand is connected to avidin by a biotin group.

20. A method as in claim 17 wherein the anti-ligand is a monoclonal antibody.

21. A method as in claim 17 wherein the surface is glass or silica.

22. A method as in claim 17 wherein the photoactivatable biotin molecule has the formula:

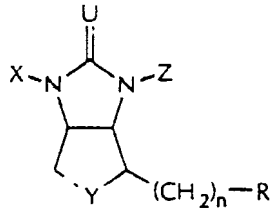


wherein X is selected from the group consisting of oxycarbonyls of lower alkyl, aryl, and benzyl groups, provided that X is not methyl oxycarbonyl; R is selected from the group consisting of hydrogen, lower alkyl, aryl, benzyl, and N-succinimidyl; and acid addition salts of the molecule.

23. A method as in claim 17 wherein the anti-ligand is attached to egg avidin.

24. A method as in claim 17 wherein the biotin analog and avidin have an affinity constant ($K_a$) of about $10^{15}$ $M^{-1}$.

25. A compound having the formula:



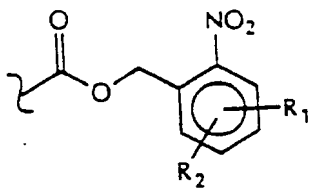
wherein X and Z are selected from the group consisting of hydrogen and oxycarbonyls of lower alkyl, aryl, and benzyl groups, provided that when X is hydrogen, Z is not hydrogen or methyloxycarbonyl, and provided that when Z is hydrogen, X is not hydrogen or methyloxycarbonyl; R is selected from the group consisting of hydrogen, lower alkyl, aryl, lower alkyl formate, aryl formate, formamide, N-alkylformamide, N-succinimidyl, hydroxyl, alkoxyl, thiol, thioether, disulfide, hydrazide and

amine groups, provided that when X or Z is methyloxycarbonyl, R is not methyl formate; U is O, S, or NH; Y is selected from the group consisting of sulfur, oxygen, methylene, carbonyl, sulfinyl and sulfonyl groups or Y represents two hydrogen atoms attached to the respective carbons; n = 0-7; and acid addition salts of the compound.

26. A compound as in claim 25 wherein U is O, Y is sulfur, and n = 4.

27. A compound as in claim 25 wherein one of X and Z is a nitroveratryloxycarbonyl group.

28. A compound as in claim 25 wherein one of X and Z has the formula:




wherein R1 and R2 are selected from the group consisting of hydrogen, lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido and phosphido groups.

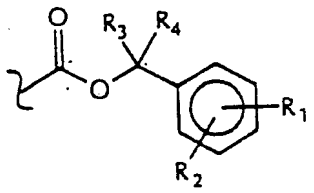
29. A compound as in claim 25 wherein X is 6-nitroveratryloxycarbonyl; Z is hydrogen; and R is methyl formate.

30. A compound as in claim 25 wherein X is 6-nitroveratryloxycarbonyl; Z is hydrogen; and R is p-nitrophenyl formate.

31. A compound as in claim 25 wherein X is 6-nitropiperonyloxycarbonyl, Z is hydrogen; and R is p-nitrophenyl formate.

32. A compound as in claim 25 wherein one of X and Z is a ring-disubstituted benzyloxycarbonyl group.

33. A compound as in claim 32 wherein the ring-disubstituted benzyloxycarbonyl group has the formula:

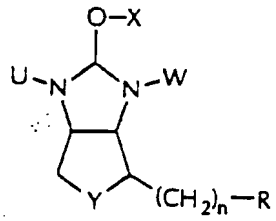


wherein $R_1$, $R_2$, $R_3$, and $R_4$ are selected from the group consisting of hydrogen, lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido and phosphido groups.

34. A compound as in claim 33 wherein $R_1$ and $R_2$ are methoxy.

35. A compound as in claim 33 wherein $R_3$ and $R_4$ are methyl.

36. A compound having the formula:

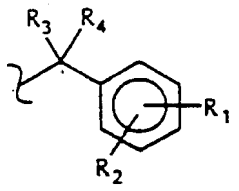


wherein X is selected from the group consisting of hydrogen, lower alkyl, aryl, and benzyl groups; U and W are selected from the group consisting of hydrogen, lower alkyl, aryl, and benzyl groups, provided that only one of U and W is present; R is selected from the group consisting of hydrogen, lower alkyl,

aryl, carboxylate, alkyl formate, aryl formate, formamide, N-alkyl formamide, N-succinimidyl, hydroxyl, alkoxyl, thiol, thioether, disulfide, hydrazide, and amine groups; Y is selected from the group consisting of sulfur, oxygen, methylene, carbonyl, sulfinyl and sulfonyl groups, or Y represents two hydrogens attached to the respective carbons; n = 0-7; and acid addition salts of the compound.

37. A compound as in claim 36 wherein Y is sulfur and n = 4.

38. A compound as in claim 36 wherein the ring-disubstituted benzyl group has the formula:



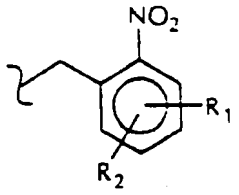

wherein $R_1$ and $R_2$ are selected from the group consisting of hydrogen, lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido and phosphido groups.

39. A compound as in claim 38 wherein $R_1$ and $R_2$ are methoxy.

40. A compound as in claim 38 wherein $R_3$ and $R_4$ are methyl.

41. A compound as in claim 36 wherein X is a nitroveratryl group.

42. A compound as in claim 36, wherein X has the formula:




wherein $R_1$ and $R_2$ are selected from the group consisting of hydrogen, lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxyl, thiol, thioether, amino, nitro, carboxyl, formate, formamido and phosphido groups.

43. A compound as in claim 36 wherein X is 6-nitroveratryl; U or W is hydrogen; and R is methyl formate.

44. A compound as in claim 36 wherein X is 6-nitroveratryloxycarbonyl hydrogen; and R is p-nitrophenyl formate.

45. A compound as in claim 36 wherein X is 6-nitropiperonyloxycarbonyl and U or W is hydrogen.

46. A compound as in claim 36 wherein X is a ring-disubstituted benzyl group, and R is methyl formate or p-nitrophenyl formate.

47. A composition comprising a compound of claim 25 covalently attached to a glass or silica surface.

48. A method for localizing a plurality of anti-ligands having different ligand binding specificities on a surface comprising the steps of:

a) attaching caged binding members to the surface;

b) exposing the caged binding members on a first predefined region of the surface to an energy source, thereby

forming binding members on the first predefined region having a strong affinity for a specific binding substance;

c) reacting the binding members on the first predefined region with the specific binding substance;

d) washing the surface to remove unbound specific binding substance;

e) exposing the surface to an anti-ligand;

f) washing the surface free of unbound anti-ligand; and

g) repeating steps (b-f) on a different region of the surface with a different anti-ligand.

49. A method for localizing a plurality of anti-ligands having different ligand binding specificities on a surface comprising the steps of:

a) attaching caged binding members to the surface;

b) exposing the caged binding members on a first predefined region of the surface to an energy source, thereby producing binding members on the first predefined region having a strong affinity for a specific binding substance;

c) reacting the binding members on the first predefined region with an anti-ligand attached to a specific binding substance;

d) washing the surface to remove unbound anti-ligand; and

e) repeating steps (b)-(d) on a different region of the surface with a different anti-ligand.

50. A method according to claim 48 wherein the energy source is ultraviolet light.

51. A method for screening a plurality of anti-ligands localized on predefined regions of a surface for affinity for ligands in solution comprising:

a) exposing the surface to a solution containing at least one marked ligand;

b) washing the surface free of unbound ligand; and

c) identifying ligands binding to anti-ligands by determining regions on the surface where markers are located.

52. A method for performing a direct assay of a marked ligand in solution by measuring the ligand's affinity for anti-ligands immobilized on predefined regions of a surface comprising the steps of:

a) exposing the marked ligand to the surface; and

b) measuring an amount of the ligand attached to the predefined regions of the surface.

53. A method for performing a competitive assay of a ligand in solution by measuring the ligand's affinity for anti-ligands immobilized on predefined regions of a surface comprising the steps of:

a) incubating a solution containing one or more marked ligands and unknown ligands with the surface;

b) washing the surface free of unbound ligands; and

c) measuring an amount of marked ligand remaining on the predefined regions of the surface.

54. A method for performing a competitive assay of a ligand in solution by measuring the ligand's affinity for anti-ligands immobilized on predefined regions of a surface comprising the steps of:

a) binding unknown ligands to anti-ligands on the surface;

b) incubating a solution containing one or more marked ligands with the surface;

c) washing the surface free of unbound ligands; and

d) measuring an amount of marked ligand remaining on the predefined regions of the surface.

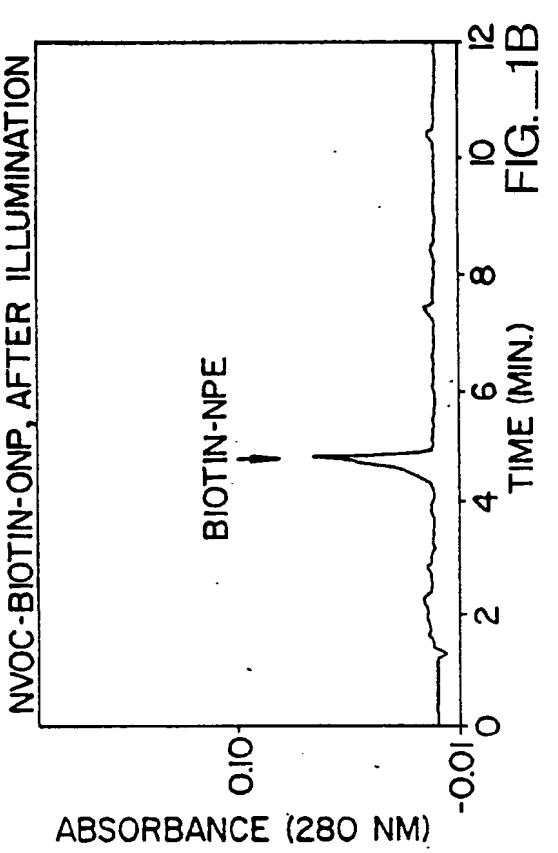



FIG._1B

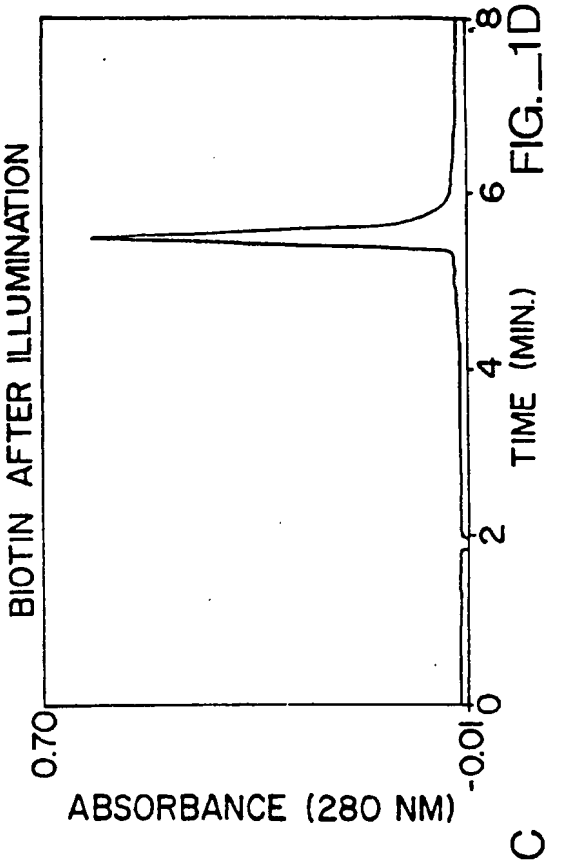



FIG._1D

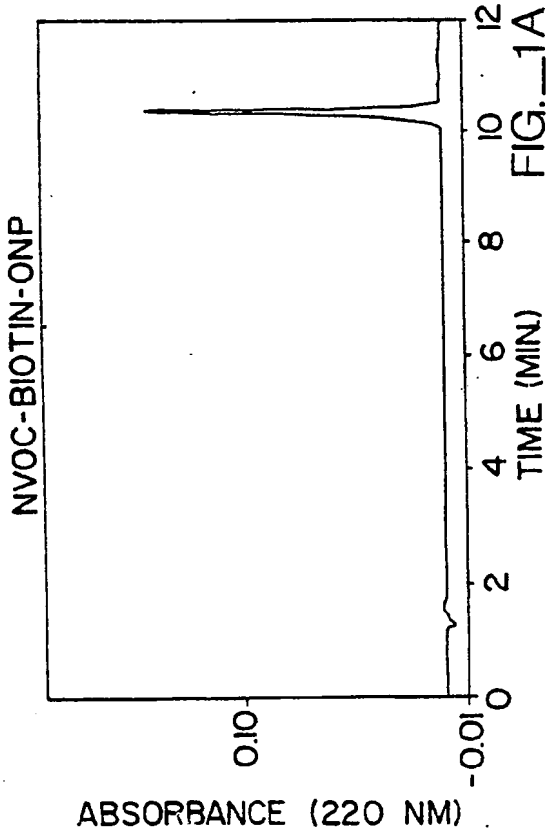



FIG._1A

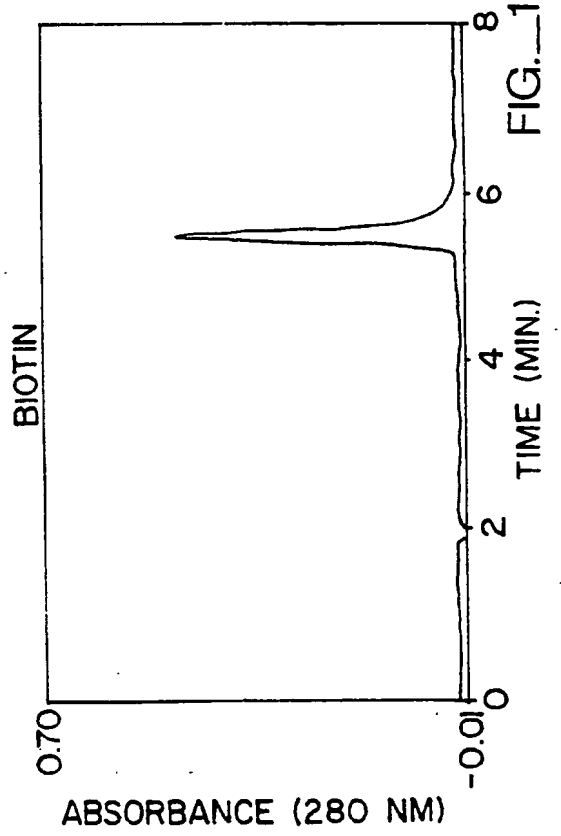



FIG._1C

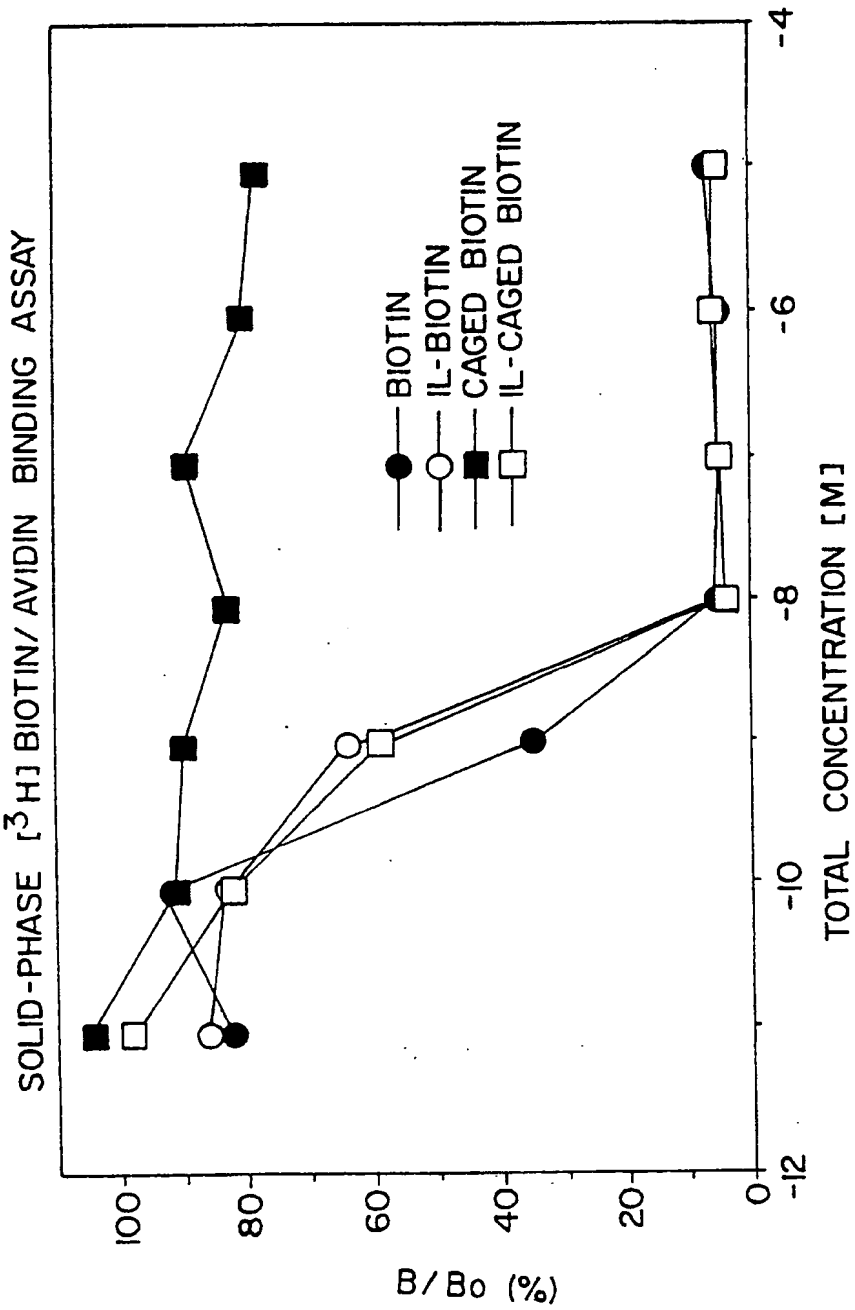

SOLID-PHASE [3H] BIOTIN/ AVIDIN BINDING ASSAY
BIOTIN
IL-BIOTIN
CAGED BIOTIN
IL-CAGED BIOTIN
B/Bo (%)
100
80
60
40
20
0
TOTAL CONCENTRATION [M]
-4
-6
-8
-10
-12

FIG._2.

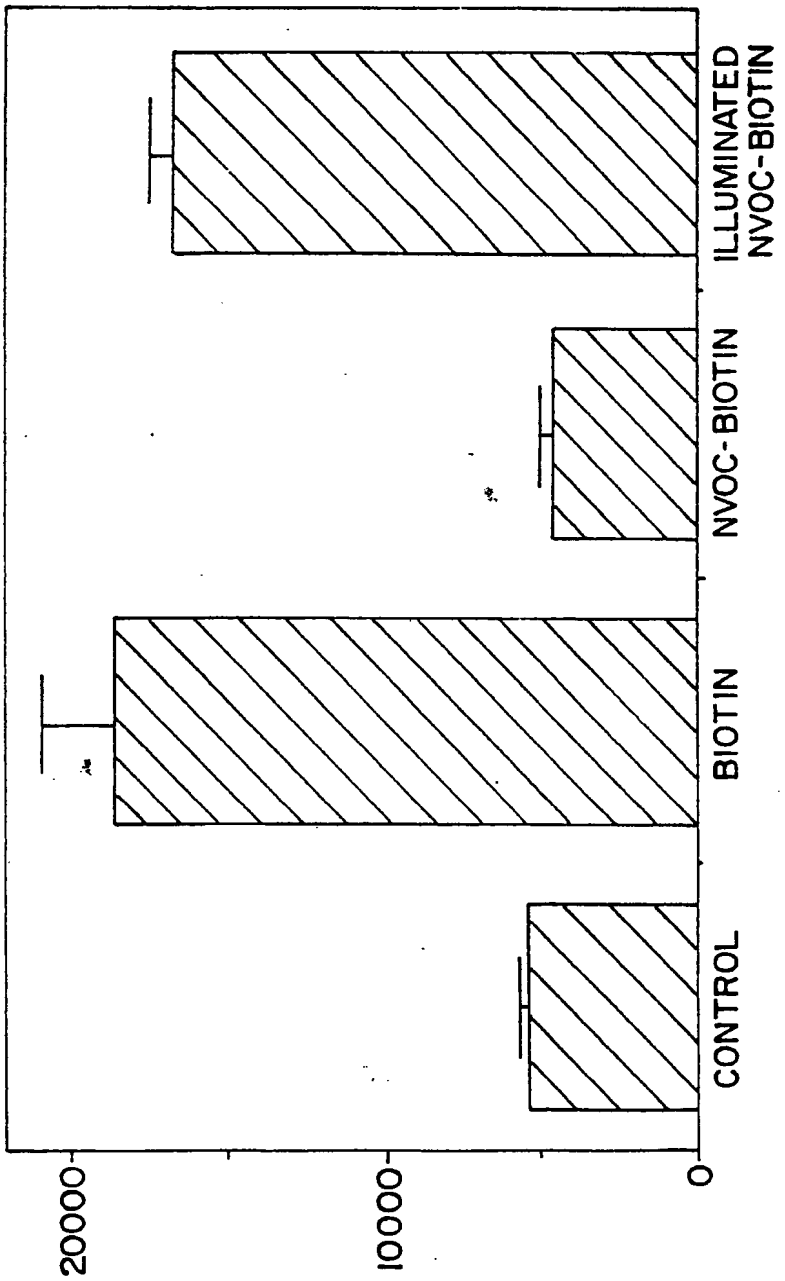

ILLUMINATED NVOC-BIOTIN
NVOC-BIOTIN
BIOTIN
CONTROL
20000
10000
0
125I- STREPAVIDIN BOUND (CPM)

FIG._3.

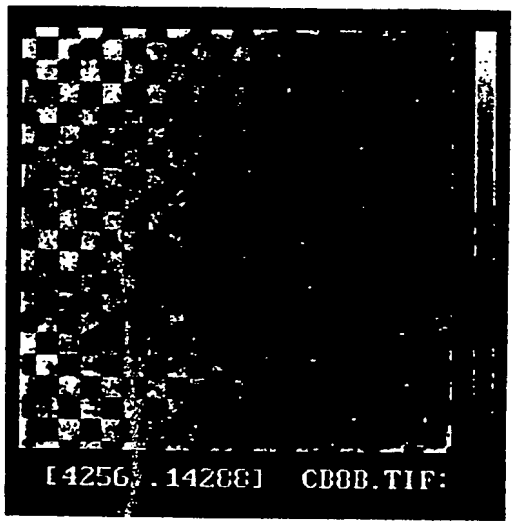



FIG._4.

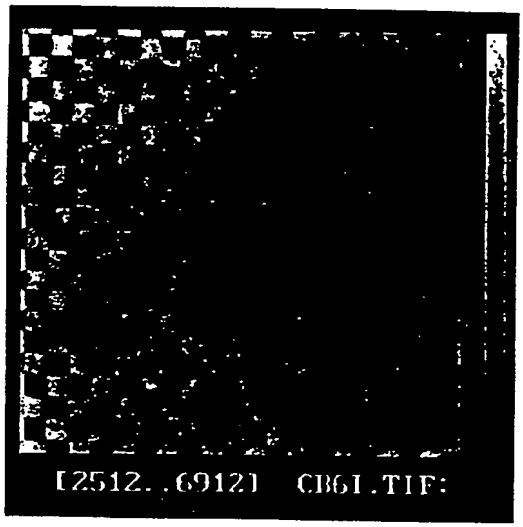



FIG._5.


FIG._6.

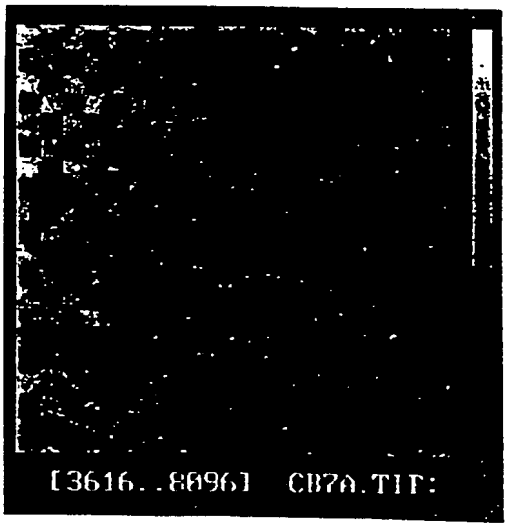



FIG._7.

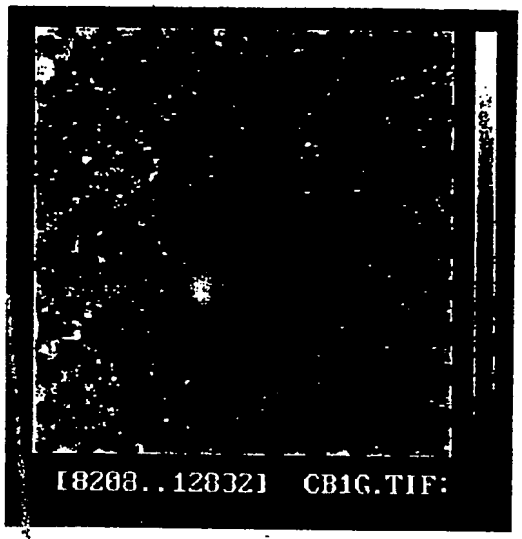

[8208..12832] CB1G.TIF:

FIG._8.

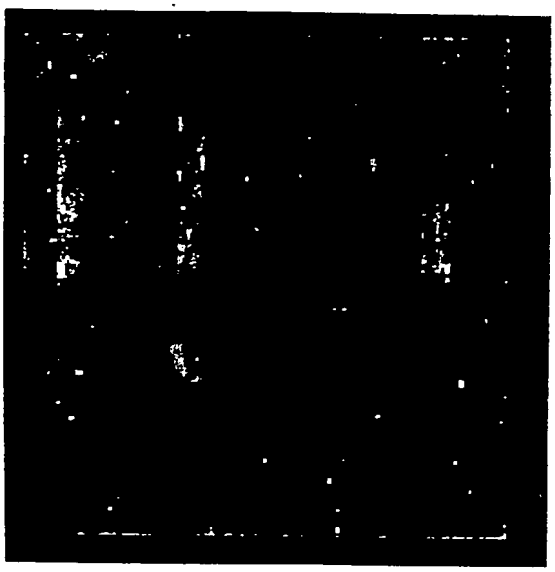

REPLICATE 1
REPLICATE 2
REPLICATE 3
REPLICATE 4
3 2 1 0
# CAPROIC ACID LINKERS

FIG._9.

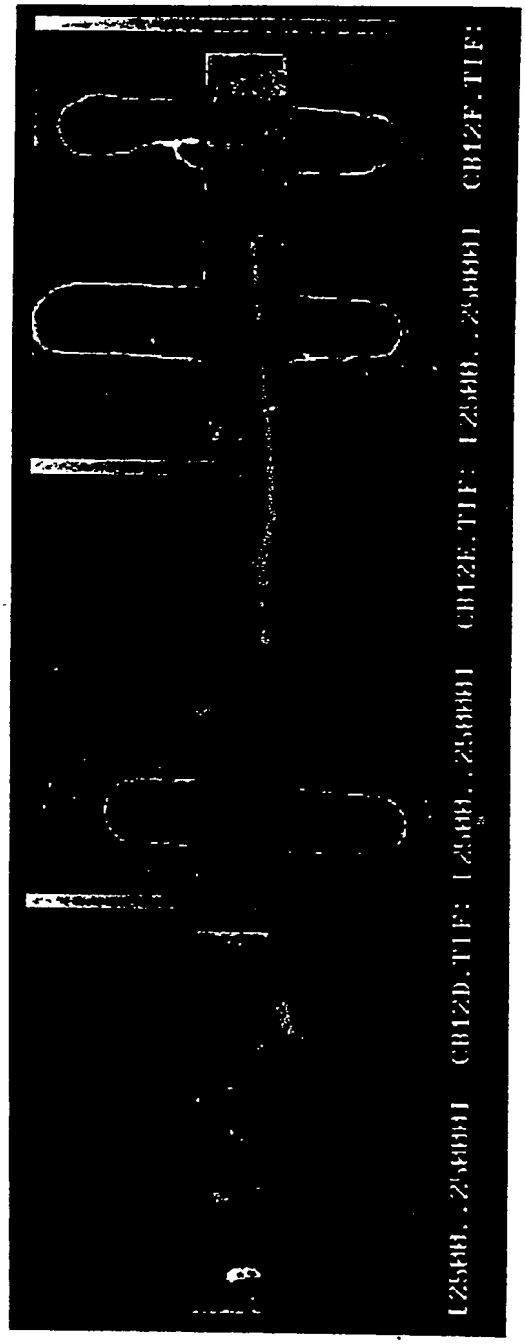



FIG._10.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/06607

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A01N 1/02; C12 Q 1/00; G01N 33/566, 33/543 ;007D 471/02,235/00,473/00,235/30

U.S. Cl.: 427/2;430/270,343,371,541;435/7.5;436/501,518,527;548/113,302,303,323

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. CL. | 427/2; 430/270, 343, 371, 541; 435/7.5; 436/501, 518, 527; 548/113, 302, 303, 323 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| X | US. A. 4.557.998 (Washburn et al.) 10 December 1985. See column 2-column 3 and column 12. lines 49-59. | 1.2.5.13. 48-50 |
| Y | US. A. 4.722.906 (Guire) 02 February 1988. See column 2-column 4. | 1-54 |
| Y | US. A. 4.567.902 (Saenger et al) 18 March 1986. See column 2. | 25-47 |
| X | US. A. 4.709.044 (Sklavounos) 24 November 1987. See column 2-column 3. | 14-35 |
| X | US. A. 4.016.043 (Schuurs et al.) 05 April 1977. See column 2-column 3. | 51-54 |
| X | US. A. 4.371.515 (Chu) 01 February 1983. See column 1-column 2. | 51-54 |
| X,P | US. A. 4.895.809 (Schlabach et al.) 23 January 1990. see entire document. | 51-54 |

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

31 January 1991

Date of Mailing of this International Search Report

11 MAR 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Jacintha Stall

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECO..D SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages. | Relevant to Claim No |
|---|---|---|
| Y | Journal Of Organic Chemistry, Volume 39, No. 2, issued 1974, Amit et al., "Photosensitive Protecting Groups of Amino Sugars and Their Use in Glycoside Synthesis. 2-Nitrobenzyloxy carbonylamino and 6-Nitroveratryloxy carbonylamino Derivatives", pages 192-196, See entire document. | 25-47 |
| Y | Journal Of The American Chemical Society, Volume 92, issued 1970, Patchornik et al., "Photosensitive Protecting Groups", pages 6333-6334, See entire document. | 25-47 |
| Y | Annual Review Of Biophysics and Biophysical Chemistry, Volume 18, issued 1989, McCray et al., "Properties and Uses of Photoreactive Caged Compounds", pages 239-270, See entire document. | 1-54 |
| A | Guire, "Methods In Enzymology", published 1976 by Academic Press (N.Y.), See pages 280-288. | 1-54 |
| A | Journal Of Organic Chemistry, Volume 42, No. 6, issued 1977, Kohn et al., "Syntheses and Spectral Properties of Substituted Imidazolidones and Imidazolines", pages 941-948. | 25-48 |
| A | Biochemical And Biophysical Research Communication, Volume 136, No. 1, issued 14 April 1986, Roffman et al., "Selective Labeling of Functional Groups on Membrane Proteins or Glycoproteins Using Reactive Biotin Derivatives and $^{125}$I-Streptavidin", pages 80-85. | 1-54 |
| A | Analytical Biochemistry, Volume 163, issued 1987, Lacey et al., "Photobiotin as a Sensitive Probe for Protein Labeling", pages 151-158. | 1-50 |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages. | Relevant to Claim No |
|---|---|---|
| A | Journal Of The American Chemical Society, Volume 100, No. 11, issued 24 May 1978, Hofmann et al., "Avidin-Biotin Affinity Columns. General Methods for Attaching Biotin to Peptides and Proteins", pages 3585-3590. | 1-54 |
| A | Journal Of Polymer Science, Volume 22, issued 1984, Ichimura, "A Convenient Photochemical Method to-Immobilize Enzymes: pages 2817-2828. | 1-50 |
| A | Biochemistry, Volume 21, issued 1982, Hofmann, et al., "Avidin Binding of Carboxyl-substituted Biotin and Analogues", pages 978-984. | 1-54 |
| A | Nucleic Acids Research, Volume 13, No. 3, issued 1985, Forster, et al., "Non-radioactive Hybridization Probes Prepared by the Chemical Labelling of DNA and RNA with a Novel Reagent, Photobiotin." pages 745-761. | 1-54 |
| A | US, A, 4,775,745 (Ford et al.) 04 October 1988. | 25-47 |
| A,P | US, A, 4,898,951 (Symons) 06 February 1990. | 25-47 |
| A | US, A, 4,656,252 (Giese) 07 April 1987. | 1-50 |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .. , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. A method for forming a predefined region on a surface of a solid support, a compound used in forming said predefined region, and a method of using said solid support classified in class 436, subclass 527 (claims 1-35 and 48-54).

II. A second compound, classified in class 548, subclass 323 (claims 36-46).

III. A third compound, classified in class 427, subclass 2. (claim 47)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.